Doutoramento em Biomedicina

Faculdade de Medicina da Universidade do Porto

Exploring the relationship between *Helicobacter pylori* and host cell invasion: from phenotypes to molecular mechanisms

Relação entre *Helicobacter pylori* e invasão das células do hospedeiro: dos fenótipos aos mecanismos moleculares

Ângela Margarida Amorim Costa

Porto, Portugal
2012

Supervisor: Prof. Céu Figueiredo (University of Porto)
Co-Supervisor: Prof. Thomas F Meyer (Max Planck Institute for Infection Biology)

Thesis committee:

Prof. Agostinho Marques (University of Porto)
Prof. Bianca Bauer (Max Planck Institute for Infection Biology)
Prof. Peter Jordan (National institute of Health" Dr. Ricardo Jorge")
Prof. Manuel Sobrinho Simões (University of Porto)
Prof. Raquel Seruca (University of Porto)
Prof. Mário Dinis Ferreira (University of Porto)
Prof. Céu Figueiredo (University of Porto)

On the front cover: *Helicobacter pylori* strain SS1. *Adapted by permission from PLoS One (Marta Correia et al.) © 2012.*

Financial support by:

SFRH/BD/36013/2007

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MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

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Este trabalho é dedicado à Célia e ao Ricardo, por me terem empurrado para a frente quando eu queria ter ficado pelo caminho

“ Nunca me resignaria na profissão ao absurdo constante de semelhantes situações catastróficas, em que a teimosia de um micróbio ou a rebeldia de uma célula desafiavam caprichosamente todas as forças mobilizadas do engenho humano.”

Miguel Torga

in “A Criação do Mundo IV”

Um doutoramento é muito mais que a tese ou a defesa, é um conjunto imenso de alegrias e frustrações que escritas dariam um livro bem maior que este. Para que o meu percurso de estudante de doutoramento (e de pré-estudante de doutoramento como BIC nos Pyloris) chegasse a bom termo tive a ajuda de muita gente, a quem tenho a obrigação de agradecer!

Desde logo gostava de agradecer a toda a comunidade do IPATIMUP, e ao seu Director, Professor Sobrinho, por ter tornado tudo isto possível. Durante estes anos o IPATIMUP foi a minha segunda casa, não só pela quantidade de tempo lá passada, mas sobretudo por sentir que faço parte de uma Família. Podemos não publicar na Sience e na Natura (ainda…), mas temos os melhores colegas e ambiente de trabalho que se pode encontrar. Desde logo um agradecimento especial ao Pessoal do Cancer Genetics por proporcionarem diariamente aquela dose de insanidade mental necessária para manter a sanidade!

Queria deixar um agradecimento muito especial à minha orientadora Céu, por me ter dado a oportunidade de trabalhar no seu Grupo, e aí fazer o meu trabalho de doutoramento. Obrigada pelas discussões científicas, por todo o teu apoio, por aturares o meu mau feitio, e por compreenderes que por detrás de um cientista está sempre uma pessoa “normal”. Céu és uma pessoa que admiro muito.

A todos os meus coleguinhas do grupo do *Helicobacter*, os de hoje e os de ontem, pela amizade e pelos conhecimentos científicos, uma muito obrigada! Um agradecimento à Ana Costa por me ter recebido no Grupo, ao Nuno Guimarães um muito obrigada especial por desencantares os artigos a que mais ninguém tem acesso, e também pelas descobertas não científicas, mas não menos espectaculares, de músicas que não lembram nem ao Menino Jesus. À Ana Machado que apareceu como colega e foi para as Dinamarcas como amiga, ao Rui pela paciência, apoio, disponibilidade com a bicheza e por me ter ensinado a fazer Real-times. À Marina pelas discussões científicas. E aos caçulas Miguel e Inês por me aturarem nos últimos tempos. E agora um agradecimento mesmo muito especial às ex-pyloris e agora inebianas Maria e Marta! À Maria porque para além de ser colega é também amiga, e é uma pessoa extraordinária com quem podemos aprender a ser melhores pessoas e cientistas, e porque é a minha mãe científica na Biologia Celular. Muito do que sou no laboratório devo-o a ti. Um obrigado também por conseguires ver coisas para além do óbvio e conseguires entusiasmar as pessoas com resultados que à primeira vista parecem uma caca. À Marta tenho a

agradecer a ajuda das suas mãozinhas mágicas quando foram precisa (obrigada por me tomares conta dos 500 mil clones do shRNA quando estava de férias). Mas sobretudo tenho a agradecer a amizade, o ombro amigo, e especialmente a paciência com que me foste aturando. Mesmo não tendo trabalhado directamente contigo nas “minhas coisas” foste sempre uma peça essencial para manter o equilíbrio e conseguir chegar ao fim desta jornada. Muito obrigada mesmo.

Queria também agradecer a todos os co-autores dos artigos que fazem parte desta tese, pelo trabalho e sugestões!

Queria agradecer à Babi, Joana Carvalho, Renata e Hugo Pinheiro pela ajuda com as coisas dos shRNA, vectores, clonagens e bicharada necessária. E às meninas da Mama: Babi outra vez, Diana e Madalena pela ajuda com as iminohistoquímicas. Um agradecimento à doutora Fátima pela ajuda com as lâminas, e, é claro, um obrigado especial à Dina pela paciência e disponibilidade.

A todas as pessoas que já fizeram e ainda fazem parte do Cancer Genetics um muito obrigado. Desde logo aos PI Raquel Seruca, Zé Carlos, Carla Oliveira e Joana Paredes por estarem sempre disponíveis quando eram solicitados. E um obrigado aos coleguinhas cá de baixo, os melhores do mundo, por fazerem com que o ambiente fosse espectacular, dentro e fora do lab. Um muito obrigado par o pessoal do Cancer Genetics que ainda não referi: Ana Sofia, André Vieira (obrigada pelas ajudas informático-burocráticas), Sílvia Carvalho, Ritinha, Nair, António Carlos, Gonçalo, Marta Correia e Carlos. E um agradecimento especial à Cecilia pela correcção didáctica do inglês da tese (que paciência!). Um obrigado muito grande aos vizinhos e ex-vizinhos da frente: Joaninha, Sérgia, Lara, Cirnes, Soraia, Bic, Xu, Sofia, Patrícia Carneiro, Margarida, Cristina e Marta Teixeira Pinto. E claro um agradecimento ao pessoal da ponta do lab Patrícia Oliveira, Mafalda, Denisa e Verónica. E um agradecimento em particular ao pessoal do Taizé pelas jantaradas no Piolho e por me acompanharem no recarregar de baterias.

E aos meus colegas do IPATIMUP extra Cancer Genetics um obrigado muito grande: à Isabel, companheira de jornada desde o inicio e ao Filipe (obrigada por ajudares a resolver os problemas informáticos), à Dina, Catarina Salgado, Hugo Prazeres, e à Joana Silva pela partilha de problemas. E um agradecimento às inebianas Ana Pinto e

Marta Oliveira por me irem recebendo no seu lab, quando queria ou precisava de “ir dar um passeio”.

E como não podia deixar de ser, um agradecimento às pessoas que fazem tudo funcionar: Mafalda, Nuno Mendes, Sr. Mário, Sr. Oliveira, Sr. Mendes, Zezinha e Cátia, pessoal da Secretaria e Informática.

A very big thank to the Berliners Yura and Malvika for the support in the lab, and to Professor Meyer for the opportunity of being part of the team. A special thank for Tim, Nicola, Manuela and Charlotte and for the extra Max Planck friends Sandra and Inês, Francesca and Pritesh.

Um agradeciemnto especial aos meus amigos da FCUP que me foram acompanhando neste caminho. Um muito obrigada à Ana bela, Helena, Virgínia, Zé, Sandro, Cláudia e Manel.

Um agradecimento muito grande à trupe do Renascer: Serginho, Paulinho, Ana, Litos, Susana, Kika, Fabricio, Gisela, Zé, Marta, Sofia, Berto, Ju, Bruno e Ruizinho. Um beijinho muito grande à Beta e à Natália pela companhia nos terapêuticos passeios a pé.

Um muito obrigada à minha Família porque sempre me deu o seu apoio incondicional, mesmo sem saber muito bem do que consta uma célula, e não compreendendo muito bem essa coisa da investigação. Um beijo muito grande ao meu Pai, à minha Mãe e à Tia Nide.

E uma palavra especial para a minha maninha, que também se quis meter na ciência e me compreende como irmã e como cientista. Não me ajudando directamente no trabalho tiveste uma importância enorme para que este trabalho se finalizasse. Muito obrigada por não me teres deixado desistir.

E a última palavra vai para o meu Amigo mais que especial e companheiro de jornada, Ricardo, porque acompanhaste o processo desde o início, porque estiveste sempre ao meu lado nos bons e nos maus momentos. Porque sem teres noção do que é uma via sinalização celular nem saberes a diferença de uma célula eucariótica de uma procariotica, tiveste aquele saber necessário para ter a palavra certa no momento exacto. É também muito por tua causa que estou hoje aqui. Obrigada por me teres empurrado para a frente.

De acordo com o Artigo 8º do Decreto-Lei nº388/70, fazem parte integrante desta dissertação os seguintes trabalhos publicados ou em publicação:

I. **Costa AM**, Ferreira RM, Oliveira MJ, Carneiro F, Leite M, Figueiredo C. *Helicobacter pylori CagA activates matrix metalloproteinase-10 in gastric epithelial cells through ERK and JNK-mediated pathways.* In preparation.

II. **Costa AM *,** Oliveira MJ *, Costa AC, Ferreira RM, Sampaio P, Machado JC, Seruca R, Mareel M, Figueiredo C. *CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses Helicobacter pylori-induced cell-invasive phenotype.* Journal of Infectious Diseases 2009 Sep 1; 200(5):745-55.
* co-autoria principal partilhada

III. **Costa AM**, Leite M, Figueiredo C. *Adherens junctions as targets of microorganisms: a focus on Helicobacter pylori.* Submitted for publication.

IV. Paredes J, Figueiredo J, Albergaria A, Oliveira P, Carvalho J, Ribeiro AS, Caldeira J, **Costa AM**, Simões-Correia J, Oliveira MJ, Pinheiro H, Pinho SS, Mateus R, Reis CA, Leite M, Fernandes MS, Schmitt F, Carneiro F, Figueiredo C, Oliveira C, Seruca R. *Epithelial E- and P-cadherins: Role and clinical significance in cancer*. Biochimica et Biophysica Acta - Reviews on Cancer 2012 May 19; 1826(2):297-311.

Em cumprimento do disposto no referido Decreto-Lei a aluna declara que participou activamente na recolha e estudo do material incluído em todos os trabalhos acima referidos. A dissertação inclui também resultados ainda não publicados. Os artigos III e IV foram parcialmente usados na elaboração da Introdução desta dissertação.

NOTA EXPLICATIVA

A presente dissertação foi escrita em Inglês na sua quase totalidade devido ao facto de alguns dos trabalhos terem sido realizados em colaboração internacional.

Abl	Abelson leukemia viral oncogene
ADAM10	A desintegrin and metalloproteinase domain-containing protein 10
AJ	adherens junction
Ala	alanine
APC	adenomatous polyposis coli
ATCC	American type culture collection
BabA	blood-group antigen-binding protein A
cagA	cytotoxin-associated gene A
cag PAI	*cag* pathogenicity island
cDNA	complementary DNA
Ck1α	casein kinase 1α
cPLA2	cytosolic phospholipase A2
Csk	c-Src tyrosine kinase
C-terminal	carboxylic terminal
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle´s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dvl	dishevelled
E-cadherin	epithelial cadherin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr-1	early growth response protein 1
EHEC	enterohemorrhagic *Escherichia coli*
EMT	epithelial to mesenchymal transition
EPEC	enteropathogenic *Escherichia coli*
ERK	extracellular signal regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
Glu	glutamic acid

GPCR	G-protein-coupled receptors
Grb2	growth factor receptor-bound protein 2
GSK3β	glycogen synthase kinase 3β
GTP	guanosine-5'-triphosphate
hBD3	human beta-defensin 3
HB-EGF	heparin binding epidermal growth factor
HER	human epidermal growth factor receptor
HGF	hepatocyte growth factor
H. pylori	*Helicobacter pylori*
HRP	horseradish peroxidase
HtrA	high temperature requirement A
IF	immunofluorescence
IFN-γ	interferon γ
IGFBP	insulin-like growth factor binding protein
IHC	immunohistochemistry
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
Ile	isoleucine
IP	immunoprecipitation
IQGAP	IQ motif containing GTPase activating protein
JAM	junctional adhesion molecule
JNK	c-Jun N-terminal kinase
Kb	kilobase
kDa	kiloDalton
LIME	Lck-interacting membrane kinase
LRP6	low density lipoprotein receptor-related protein 6
MALT	mucosal-associated lymphoid tissue
MLC	myosin light chain
MMP	metalloproteinase
MOI	multiplicity of infection
mRNA	messenger RNA
MUC	mucin
MW	molecular weight
N-cadherin	neuronal cadherin

NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nod1	nucleotide-binding oligomerization domain protein 1
NTAL	non-T cell activation linker
N-terminal	amino terminal
PDFG	platelet derived growth factor
PDFGR	platelet derived growth factor receptor
PI3K	phosphatidylinositol 3-kinase
PLC-γ	phospholipase C γ
Pro	proline
PTP1B	protein tyrosine phosphatase 1B
OipA	outer inflammatory protein A
PBS	phosphate buffered saline
PKC	protein kinase C
PP1α	phosphoprotein phosphatase 1α
P-Tyr	phospho tyrosine
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RTK	receptor tyrosine kinase
qRT-PCR	quantitative real time polymerase chain reaction
SabA	sialyl-Lewis X binding protein A
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	standard error
SF	scatter factor
SH2	Src homology 2
siRNA	small interference RNA
STAT3	signal transducer and activator of transcription 3
T4SS	type IV secretion system
TCF-LEF	T-cell factor-lymphoid enhancer factor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
Tir	Translocated intimin receptor
TNF-α	tumor necrosis factor α
TSA	trypticase soy agar

Tyr	tyrosine
vacA	vacuolating cytotoxin gene A
WB	western blot
ZO-1	zonula occludens

INTRODUCTION

1. *Helicobacter pylori*

Helicobacter pylori (*H. pylori)* is a spiral shaped microaerophilic Gram-negative bacterium that colonizes the human stomach (Figure 1). These bacteria were first isolated from human stomachs of patients with gastritis and peptic ulcers in 1983 (Marshall *et al.*, 1984), and are thought to have coevolved with humans for thousands of years (Ghose *et al.*, 2002, Falush *et al.*, 2003). *H. pylori* infects more than half of the human population worldwide, with the highest rates of infection in developing countries (Rothenbacher *et al.*, 2003). The infection is acquired mainly in childhood and is transmitted from person to person mainly by fecal-oral, gastro-oral and oral-oral routes (Azevedo *et al.*, 2007), persisting throughout the host lifetime if not treated. One single individual may be colonized with different *H. pylori* strains, as well as with variants of those strains (Kuipers *et al.*, 2000).

In the gastric mucosa, *H. pylori* can be found within the mucus layer and in close contact with the epithelial cells, preferentially at the apical side of the intercellular contacts (Hazell *et al.*, 1986, Necchi *et al.*, 2007, Tan *et al.*, 2009). Although not so frequently described in the literature, it has also been observed that *H. pylori* reaches the intercellular spaces below the tight junctions, and even penetrates the epithelium towards the *lamina propria* in the basolateral side of the cells (Necchi *et al.*, 2007), showing that *H. pylori* is able to disrupt the adherens junctions. Interestingly, there are also reports demonstrating the presence of *H. pylori* inside the host cells, both in biopsies and in *in vitro* models (Noach *et al.*, 1994, el-Shoura, 1995, Engstrand *et al.*, 1997, Wilkinson *et al.*, 1998, Su *et al.*, 1999, Petersen *et al.*, 2000, Necchi *et al.*, 2007).

The colonization of the stomach by *H. pylori* alters the gastric epithelial tissue since the bacteria use host resources and produce toxins that may function as signals to the eukaryotic cells.

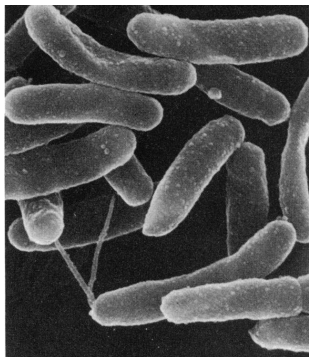


Figure 1. Electron micrograph of *H. pylori*, a Gram-negative spiral-shaped bacterium with flagella that confer motility. Adapted by permission from *Antimicrobial Agents and Chemotherapy (Iwahi et al.) © 1991.*

1.1. *H. pylori* virulence factors

H. pylori has different virulence factors that allow bacteria to colonize an aggressive habitat as the human stomach, with very low pH. Examples of virulence factors are the flagella, which allow the bacteria to move through the mucus layer of the stomach, and the urease enzyme that is able to catalyze urea into carbon dioxide and ammonia, and, in that way, increase the gastric pH. Other virulence factors are the BabA and SabA adhesins, important for the bacteria adhesion to the epithelial cells, and OipA, an outer membrane protein that contributes to the inflammatory response induced by the bacteria (Kusters *et al.*, 2006).

Three of the most well studied virulence factors are VacA, CagA and the *cag* pathogenicity island (*cag* PAI), which codifies for a Type 4 Secretion System (T4SS) as well as for CagA. While the presence of T4SSs is common to other bacteria, such as *Agrobacterium tumefaciens* (Cascales *et al.*, 2003), the *cagA* and *vacA* genes do not have homologues in other species.

All the *H. pylori* strains express VacA, a multimeric high-molecular weight secreted toxin, which causes vacuole formation in epithelial cells *in vitro* (Cover *et al.*, 1992). VacA has the ability to form pores in the membrane of the host cells, allowing the release of anions and urea (Iwamoto *et al.*, 1999, Tombola *et al.*, 2001), and may have an important role in the pH regulation of the stomach. VacA is also important for the nutrient acquisition by the bacteria due to its role in loosening epithelial tight junctions (Papini *et al.*, 1998). It is also reported that VacA is involved in cytoskeleton organization, cell migration, and in apoptosis, either by vacuole formation or by its capacity to interfere with mitochondria, inducing the release of the cytochrome c, a pro-apoptotic signal (Pai *et al.*, 1999, Kuck *et al.*, 2001, Cover *et al.*, 2003, Galmiche *et al.*, 2000) (Figure 2). The gene encoding VacA has three different variable regions: s (signal), i (intermediate) and m (middle), with two allelic types in each region. The VacA s1/i1/m1 combination is more frequently observed in strains associated with disease (Atherton *et al.*, 1995, van Doorn *et al.*, 1998, Nogueira *et al.*, 2001, Rhead *et al.*, 2007, Basso *et al.*, 2008).

The *cag* PAI is a 40 Kb region that encodes a T4SS and is present in about 60% of *H. pylori* strains in Western countries (Censini *et al.*, 1996). The T4SS system functions like a needle through which the bacteria can inject effector molecules into the host cell. The only two recognized effectors translocated by the T4SS are the CagA toxin and peptidoglycan components (Odenbreit *et al.*, 2000, Viala *et al.*, 2004). The latter are recognized by the Nod1 receptor, leading to the activation of the NF-kB pathway and to increased IL-8 production by the host cell (Viala *et al.*, 2004). Either through its effectors

or by the contact of the structure with epithelial cells the T4SS induces several alterations in the cellular signaling machinery of the host, such as the transcription of pro-apoptotic and pro-inflammatory mediators (Viala et al., 2004). It has also been reported that strains which lack the *cag* PAI have little interaction with the host cells (Guillemin et al., 2002)

The CagA effector, after injection into the host cell localizes close to the cell membrane (Segal *et al.*, 1999, Asahi *et al.*, 2003). CagA can be tyrosine phosphorylated by c-Src and c-Abl kinases (Selbach *et al.*, 2002, Tammer *et al.*, 2007) at conserved EPIYA (Glu-Pro-Ile-Tyr-Ala) motifs (Hatakeyama, 2004). Both phosphorylated and non-phosphorylated CagA are able to interact with several host proteins. Upon phosphorylation, CagA is able to interact with Csk, FAK, and SHP-2 leading to increased cell proliferation, cytoskeletal reorganization and motility (Higashi *et al.*, 2002, Tsutsumi *et al.*, 2003, Tsutsumi *et al.*, 2006)(Figure 2). Non-phosphorylated CagA interacts with c-Met, Grb2, PAR-1, and ZO-1. These interaction with the host signaling molecules result in cytoskeleton rearrangements, named “hummingbird phenotype,” characterized by spreading and elongation of the host cells, and in junctional and polarity defects (Mimuro *et al.*, 2002, Amieva *et al.*, 2003, Churin *et al.*, 2003, Saadat *et al.*, 2007, Segal *et al.*, 1999) (Figure 2).

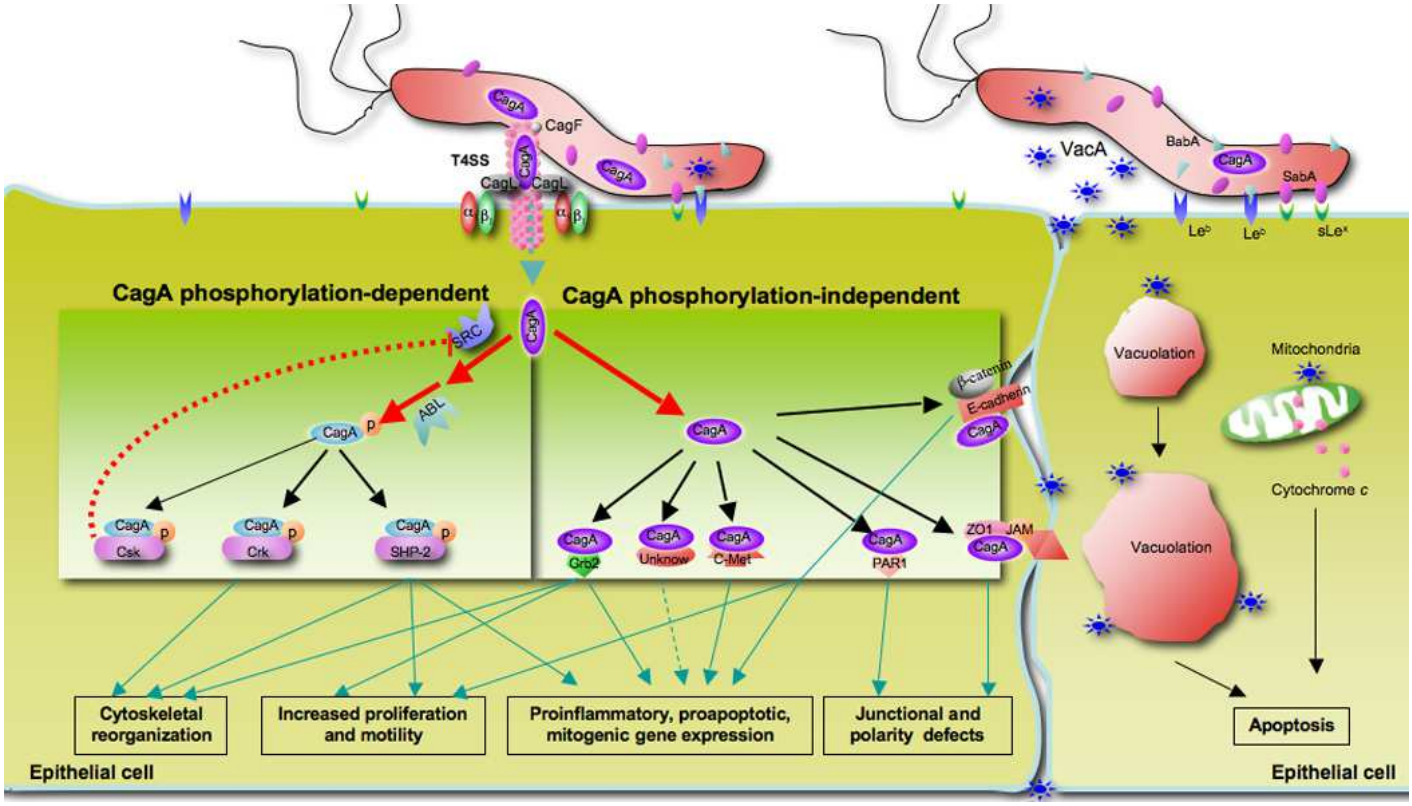


Figure 2. Overview of the CagA, T4SS and VacA-induced host cell alterations. *Adapted by permission from Cancer Letters (Wen and Moss) © 2009.*

1.2. Clinical outcomes of *H. pylori* infection

H. pylori infection induces an inflammatory process that results in the establishment of chronic superficial gastritis. In the initials steps of *H. pylori* colonization, both innate and acquired immune systems are activated (Peek *et al.*, 1995), but with the chronicity of the infection the immune stimulation remains constant, with the levels of antibodies remaining constant for 20 years (Perez-Perez *et al.*, 2002). The continuous stimulation of the immune system results in the infiltration of neutrophils and lymphocytes into the gastric mucosa, and in the production of cytokines by the host cells. The response is more pronounced in the case of *cag* PAI positive strains (Crabtree *et al.*, 1991a, Peek *et al.*, 1995, Atherton *et al.*, 1996). It is described that *H. pylori* up-regulates pro-inflammatory cytokines such as IL-8, IL-6, TNF-α, INF-γ, TGF-β, as well as anti-inflammatory cytokines like IL-10 (reviewed by (Romero-Adrian *et al.*, 2010).

In the majority of the infected individuals, *H. pylori* infection results in chronic superficial gastritis only. However, in some individuals the infection can lead to more severe diseases such as peptic ulcer disease, gastric mucosal-associated lymphoid tissue (MALT) lymphoma, or gastric carcinoma.

1.2.1. *H. pylori*-associated gastric carcinoma

The World Health Organization has considered *H. pylori* as a type I carcinogen. This is particularly relevant since recent reported data revealed that about 16% (2 million cases) of cancers registered in the world per year, are attributable to infections (de Martel *et al.*, 2012), in which *H. pylori*, hepatitis B and C viruses, and human papillomaviruses were responsible for 1.9 million cases .

H. pylori is associated with the two main histological types of gastric cancer: intestinal and diffuse (Nomura *et al.*, 1991, Correa, 1992, Uemura *et al.*, 2001). However, in the case of the intestinal type the mechanisms of *H. pylori* induced gastric cancer are better characterized. The bacteria function as an initiator factor of a cascade that begins with chronic superficial gastritis, and evolves to atrophic gastritis, characterized by loss of the glands that produce the gastric acid, and by infiltration of inflammatory cells in the glandular zone. Some individuals with atrophic gastritis may develop intestinal metaplasia, a premalignant lesion characterized by the substitution of the gastric cells by cells with intestinal markers. Intestinal metaplasia can evolve to dysplasia and finally to gastric carcinoma (Correa, 1992, Suerbaum *et al.*, 2002) (Figure 3).

There is a considerable amount of data pointing to the role of *H. pylori* in the establishment of gastric carcinoma. Experiments with Mongolian gerbils where among the first demonstrate that the infection with *H. pylori* results in the development of gastric carcinoma (Watanabe *et al.*, 1998). Studies in humans also showed that in patients with no precancerous lesions, gastric carcinoma only arose in *H. pylori*-infected individuals (Uemura *et al.*, 2001, Wong *et al.*, 2004). Another important indication for the importance of *H. pylori* in gastric carcinoma development are the studies that show that anti-microbial therapy directed to *H. pylori* prevented or delayed the development of gastric carcinoma (Correa *et al.*, 2000, Uemura *et al.*, 2001). In a prospective randomized placebo-controlled population study in China, enrolling 988 infected individuals without precancerous lesions, gastric carcinoma only arose in those individuals who were not *H. pylori* eradicated (Wong *et al.*, 2004). In another study, *H. pylori*-infected gastric carcinoma patients that underwent endoscopic resection of the tumor and that were either *H. pylori* eradicated or left untreated after surgery were analyzed (Uemura *et al.*, 1997). The eradication of *H. pylori* resulted in regression of precancerous lesions and no development of gastric carcinoma, while the non-eradicated group reported 9% of new early stage intestinal type gastric carcinomas (Uemura *et al.*, 1997). Although still a matter of debate, *H. pylori* eradication in some studies appears to lead to regression of gastric atrophy and intestinal metaplasia (Correa *et al.*, 2000).

Gastric carcinoma development occurs in a very small proportion of the *H. pylori*-infected individuals, and this has been attributed to variations in host, bacterial, and environmental factors. Host polymorphisms that increase the IL-1β or TNF-α inflammatory response, are associated with an increased risk of developing atrophy, hypochlorhydria, and gastric carcinoma (Machado *et al.*, 2001b, El-Omar *et al.*, 2000, El-Omar *et al.*, 2003, Machado *et al.*, 2003). Environmental factors such as cigarette smoking and diets with high salt intake increase the risk of gastric carcinoma development, whereas diets with high levels of antioxidants are protective (Hansson *et al.*, 1993). *H. pylori* virulence factors that vary among strains are also associated with differences in risk of disease. The VacA s1/i1/m1 strains cause greater damage in the epithelial cells and are associated with increased risk for gastric carcinoma (Atherton *et al.*, 1995, Kidd *et al.*, 1999, Miehlke *et al.*, 2000). In the same line, *cag* PAI positive strains induce more inflammation and pronounced effects on cell cycle and cytoskeleton organization. These constitute the majority of the strains isolated from patients with severe diseases (Crabtree *et al.*, 1995, Censini *et al.*, 1996, Segal *et al.*, 1999, Peek *et al.*, 2002).

Although association studies show that *H. pylori* (and specific virulence factors), host, and environmental factors confer increased risk for the development of gastric carcinoma, the

molecular mechanisms behind this process are not well known.

H. pylori affects the epithelium in a multistep degenerative process that starts with the inflammation associated with the gastric colonization, with relevance for the action of the interleukins and reactive oxygen and nitrogen species produced in that context. At the same time, *H. pylori* promotes epithelial damage at the site of attachment on the host cell, both by altering the cell to cell adhesion and by the production of urease, lipases and toxins. In these conditions, the subepithelial tissue and the extracellular matrix are exposed, promoting the formation of ulcers. The disruption of epithelial junctions by *H. pylori* leads to a cell scattering phenotype, and causes cell elongation and increased motility (Montecucco *et al.*, 2001). These alterations in the epithelium can lead to the disruption of the balance between epithelial cell proliferation and apoptosis (Cover *et al.*, 2003). Indeed *H. pylori* has been associated with increased host cell apoptosis and with increased proliferation (Moss *et al.*, 1996, Brenes *et al.*, 1993). Additionally, *H. pylori* infection has been associated with DNA damage. This may occur either by the direct action of *H. pylori* virulence factors or by indirect action of reactive oxygen and nitrogen species produced by inflammatory cells (Smoot *et al.*, 2000, Kim *et al.*, 2002b, Maeda *et al.*, 2002). In agreement with these observations, it has been shown that *H. pylori* downregulates mismatch repair systems of the host cells (Kim *et al.*, 2002a, Machado *et al.*, 2009).

Overall, alterations in the balance of epithelial cell apoptosis and compensatory hyperproliferation induced by *H. pylori* may render these cells more susceptible to acquire mutations which may not be repaired, and therefore have increased risk for malignant transformation.

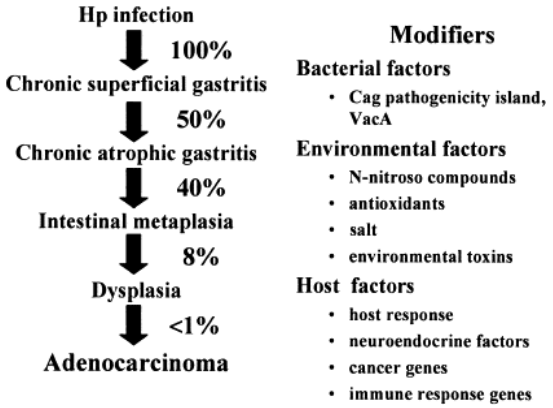


Figure 3. Host, environmental and bacterial factors involved in the cascade leading to gastric carcinoma. *Adapted by permission from Gastroenterology (Ernst et al.) © 2006.*

1.3. *H. pylori* and host epithelial cell-cell junctions

The gastric epithelium functions as a physical barrier, and seal the lumen preventing gastric acid and pathogens to reach the interstitial space and the blood stream.
Epithelial cells are polarized structures linked to each other and to the extracellular matrix by cellular junctions. The function of the epithelium is mainly accomplished by the existence of these cell-cell junctions – tight junctions, adherens junctions, desmosomes, and gap junctions – which are essential components of epithelial integrity (Moens *et al.,* Turnberg, 1985) (Figure 4). Defects of the junctional complexes are characteristic of several diseases, including cancer. The loss of cell-cell adhesion may induce loss of contact inhibition (Pignatelli *et al.*, 1994), contributing to the early phases of malignant transformation of epithelial cells.

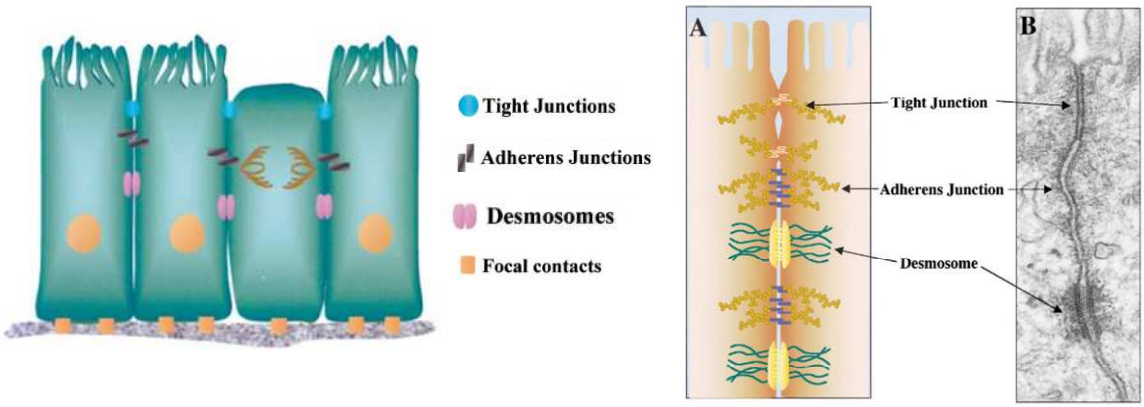


Figure 4. Organization of the intercellular junctions. Epithelia are composed of a layer of cells attached to the basement membranes by focal contacts and to the adjacent cells by tight junctions, adherens junctions, and desmosomes. *Adapted by permission from Cell (Perez-Moreno et al.) © 2003.*

The tight junctions are the most apical set of cell-cell junctions that separate the apical and the basolateral domains of the plasma membrane. Tight junctions functions, as a barrier that impairs the mixing of lipids and proteins from the apical and basolateral parts of the epithelial cells, and at the same time, control the paracellular flux of ions and small molecules (Tsukita *et al.*, 2001, Balda *et al.*, 2008). They are composed of transmembrane proteins occludin, claudins, and junctional adhesion molecules (JAMs), and cytosolic proteins, zona ocludens (ZO)-1, -2, and 3, that bridge transmembrane

proteins with the cytoskeleton (Tsukita *et al.*, 2001). It has been shown that *H. pylori* infection of epithelial cells induces internalization of occludin and claudins, and increases myosin light chain phosphorylation, leading to tight junction relaxation and increased epithelial barrier permeability (Wroblewski *et al.*, 2009, Fedwick *et al.*, 2005, Lapointe *et al.*, 2010). Further, CagA interacts with ZO-1 and JAM-A, and recruits these proteins to the sites of bacteria attachment, altering the composition and function of the tight-junctional complex (Amieva *et al.*, 2003). The VacA toxin was also described to decrease the transepithelial resistance, a process mediated by the tight junctions complexes (Papini *et al.*, 1998, Pelicic *et al.*, 1999).

Gap junctions are formed by channels constituted by connexins that allow intercellular passage of ions and small molecules such as calcium, ATP and cAMP (Laird, 2006). In addition, gap junctions also have a role in regulating cell morphology, establishing polarity, and rearrangement of the cytoskeleton (Matsuuchi *et al.*, 2012). There are few reports addressing the role of *H. pylori* in gap junction modulation. Both CagA-positive and negative strains affect the gap-junctional function, with a more significantly effect in the case of CagA-positive strains (Tao *et al.*, 2007, Xu *et al.*, 2008), and bacteria eradication leads to promotion of cell junction formation (Xu *et al.*, 2008).

Desmosomes provide mechanical stability and intercellular communication to neighboring cells. They are composed by transmembrane desmoglein and desmocollin cadherins, which bind cytoplasmatic plakoglobin and plakophilin, which in turn bind to desmoplakin. The latter anchors intermediate filaments, which establish a mechanical continuum across cells (Odland, 1958, Matter *et al.*, 2003, Johnson, 2005). The infection with *H. pylori* resulted in no alteration in the components of the desmosomes, in patients with gastroesophageal reflux disease (Wex *et al.*).

1.3.1. *H. pylori* and adherens junctions disruption

Adherens junctions are localized immediately below tight junctions and their main function is to maintain cell-cell adhesion. The major component of the adherens junctions is the transmembrane protein E-cadherin. The extracellular part of E-cadherin establishes homophilic interactions with E-cadherin molecules of neighboring cells, promoting cohesion of the epithelium. E-cadherin may also establish heterophilic interactions, namely with the receptor tyrosine kinases EGFR and c-Met (Hoschuetzky *et al.*, 1994, Mateus *et al.*, 2007, Hiscox *et al.*, 1999, Reshetnikova *et al.*, 2007), modulating their signaling properties. The cytoplasmic domain of E-cadherin is associated with β-,

p120-, and α-catenins, and the latter establishes a connection between E-cadherin and the actin cytoskeleton (Figure 5). These protein-protein interactions, as well as the phosphorylation status of the catenins, are important in junction stabilization (Reynolds *et al.*, 2004b, Yanagisawa *et al.*, 2006). The alterations in the phosphorylation status of the adherens junctions components can be mediated by RTKs such as EGFR, c-Met and FGFR or by the non-receptor tyrosine kinase c-Src, resulting in the disassembly of the cytoplasmatic adhesion complex, disruption of cadherin-mediated cell adhesion and cell scattering (Behrens *et al.*, 1993, Hamaguchi *et al.*, 1993, Fujita *et al.*, 2002).

Apart from the adhesive function, adherens junctions are important in regulation and maintenance of cellular polarity, actin cytoskeleton and organelles organization, motility, tight junction formation, signal transduction, inhibition of proliferation, prevention of apoptosis, and are important to suppress epithelial cell invasion and metastatic spread in a cancer context. In the absence of E-cadherin the other members of the complex are not able to promote cell-cell adhesion (Jamora *et al.*, 2002, Bilder *et al.*, 2000).

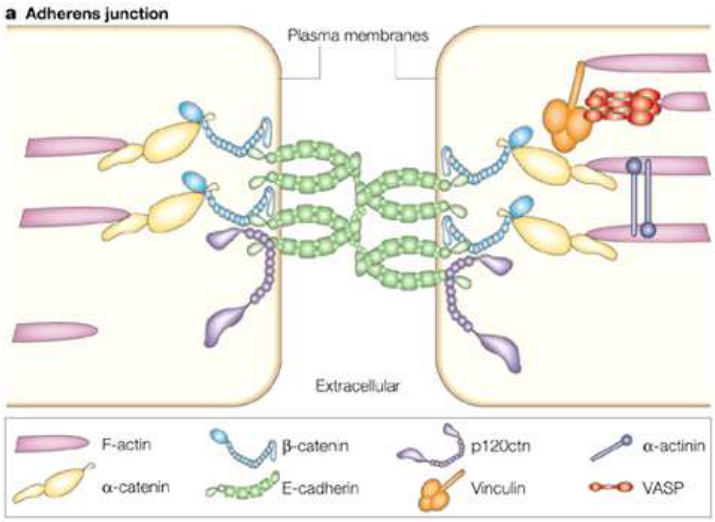


Figure 5. Schematic representation of the adherens junction complex. *Adapted by permission from Nature Reviews Genetics (Fuchs and Raghavan) © 2002.*

Disruption of the intercellular junctions is a strategy used by several microorganisms as a means of adhering to, entering cells, and/or exploiting host signaling to their advantage. Although the first line of defense against infectious agents in mucosal epithelia are tight junctions, microorganisms also explore cell-cell junctions at levels below the tight junctions. Indeed, E-cadherin is used as a receptor for adhesion and/or internalization by

several microorganisms, allowing microbial persistence in the host, avoidance of mechanical clearance, and increased pathogenesis (Mengaud *et al.*, 1996, Lecuit *et al.*, 2000, Lecuit *et al.*, 2001, Phan *et al.*, 2007, Wu *et al.*, 1998, Katz *et al.*, 2000, Inoshima *et al.*, 2011).

Several lines of evidence show that *H. pylori* interferes with cell-cell junctional complexes, although the importance of this phenomenon for gastric disease development is not fully understood.

The known mechanisms underlying *H. pylori*-mediated adherens junctions disruption are diverse and directed towards different components of the complex.

H. pylori and E-cadherin

Studies have shown that *H. pylori* targets E-cadherin by several mechanisms. Promoter methylation of the *CDH1* gene (encoding E-cadherin) is frequently observed in *H. pylori*-infected individuals (Chan *et al.*, 2003a, Leung *et al.*, 2006, Perri *et al.*, 2007). The importance of this epigenetic mechanism of gene silencing in the context of *H. pylori* infection is reinforced by studies showing that eradication of *H. pylori* reduces *CDH1* promoter methylation levels (Leung *et al.*, 2006, Perri *et al.*, 2007, Chan *et al.*, 2006). Although the molecular mechanisms through which *H. pylori* mediates E-cadherin promoter methylation are not fully understood, Qian *et al*. have shown that *CDH1* promoter methylation could be induced by infection with *H. pylori* or treatment of gastric cancer cell lines with interleukin-1β (IL-1β), a cytokine up-regulated in the context of *H. pylori* infection (Qian *et al.*, 2008). This effect was inhibited by treatment with an interleukin-1 receptor antagonist (IL-1ra) antibody, suggesting that IL-1β may play a role in E-cadherin methylation (Qian *et al.*, 2008). More recently, the same group has extended the study and showed that *H. pylori*- and IL-1β-mediated *CDH1* promoter methylation, led to decreased E-cadherin expression and concomitant increase in DNA methyltransferase activity (Huang *et al.*, 2012). Nevertheless, other studies have failed to show a decrease in E-cadherin expression associated with *H. pylori* infection both in cell lines and in the gastric mucosa (Bebb *et al.*, 2006, Conlin *et al.*, 2004, Chan *et al.*, 2003a). Of note, no *CDH1* mutations have been described associated to *H. pylori* infection.

In addition to epigenetic silencing, *H. pylori* has been associated with other mechanisms of disturbance of E-cadherin functions, such as proteolytic cleavage of its extracellular domain (also known as ectodomain shedding), and protein delocalization from the cell membrane.

Weidig *et al.* and Schirmeister *et al.* reported E-cadherin ectodomain shedding upon *H. pylori* infection in two different cell line models, the breast cancer MCF-7 and the gastric cancer NCI-N87 cells, respectively (Schirrmeister *et al.*, 2009, Weydig *et al.*, 2007). In both reports, ectodomain shedding of E-cadherin was neither dependent on *H. pylori* CagA nor on the presence of a functional T4SS. This finding is in keeping with a recent report showing that *H. pylori*-positive patients had significantly higher serum levels of soluble E-cadherin than uninfected controls, independently of the CagA status of the infecting strain (O'Connor *et al.*). There is evidence that the host disintegrin metalloproteinase ADAM10 contributes to *H. pylori*-induced shedding of E-cadherin in NCI-N87 cells, but since specific inhibition of ADAM10 led to a partial inhibition of E-cadherin shedding, it is possible that other proteases are also involved in this process (Schirrmeister *et al.*, 2009). In fact, Hoy *et al.* have recently identified the high-temperature requirement A (HtrA), a serine protease from *H. pylori*, as a new secreted virulence factor which cleaves the ectodomain of the E-cadherin. E-cadherin shedding by HtrA leads to epithelial barrier disruption and may allow *H. pylori* to access the intercellular spaces (Hoy *et al.*, 2010). HtrA-mediated E-cadherin cleavage may be a pathogenic mechanism of multiple Gram-negative bacteria, as is has recently been shown for pathogens such as enteropathogenic *Escherichia coli*, *Shigella flexneri*, and *Campylobacter jejuni* (Hoy *et al.*, 2012).

Studies have shown that *H. pylori* also induces E-cadherin translocation from the cell membrane to the cytoplasm (Schirrmeister *et al.*, 2009, Conlin *et al.*, 2004). Conlin *et al*. have demonstrated that *H. pylori*-induced redistribution of E-cadherin to intracellular vesicles was accompanied by the translocation from the cytoplasm to intracellular tubular structures of IQGAP-1, a protein that regulates the formation of adherens junctions. The results of E-cadherin internalization mediated by *H. pylori* in the NCI-N87 gastric cell line were confirmed in primary gastric cells. After 48 hours of bacterial infection, the presence of a reduced level of E-cadherin at the cell membrane was demonstrated, mediating a reduced level of cell-cell adhesion. These alterations to epithelial cell adhesion molecules induced by *H. pylori* were paralleled by increased levels of Rho-GTP and cell migration (Conlin *et al.*, 2004). Using the human breast cancer MCF-7 cell line, Weidig *et al.* also reported internalization of E-cadherin upon *H. pylori* infection, simultaneously with E-cadherin cleavage (Weydig *et al.*, 2007). A rapid dissociation of the E-cadherin/β-catenin/p120 complex from the actin cytoskeleton was observed upon infection, by disruption of the interaction between E-cadherin and α-catenin (Weydig *et al.*, 2007).

Although E-cadherin alterations mediated by *H. pylori* are independent of CagA, physical interaction between these proteins has been described (Murata-Kamiya *et al.*, 2007).

Murata-Kamiya *et al.* used the gastric cancer cell line MKN28 transfected with CagA expression vectors, and showed that CagA was able to interact with E-cadherin. This led to β-catenin release from the adherens junctions and nuclear translocation, with transactivation of genes encoding intestinal specific proteins like MUC2, contributing to the development of intestinal metaplasia (Murata-Kamiya *et al.*, 2007). This effect was independent of the CagA tyrosine phosphorylation status but dependent on a specific region of CagA known as the multimerization sequence (Kurashima *et al.*, 2008).

H. pylori and β-catenin

The binding of catenins to the intracellular domain of E-cadherin has a crucial role in the adherens junctional complex stabilization, connection to the cell cytoskeleton, and cell signaling.

When not tethered at the membrane in the adherens junctions, β-catenin is phosphorylated by a multiproteic complex comprising the serine/threonine kinase glycogen synthase kinase 3β (GSK3β), and the scaffolding proteins adenomatous polyposis coli (APC), axin, and casein kinase 1α (Ck1α) and targeted to degradation via the ubiquitin/proteasome pathway (Aberle *et al.*, 1997, Gooding *et al.*, 2004). This degradation pathway is counteracted when the Wnt pathway is active, resulting in β-catenin stabilization in the cytoplasm. Cytoplasmic stabilized β-catenin may be translocated into the nucleus where it forms a nuclear complex with the transcription factors of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family, promoting the expression of a wide-range of genes important for carcinogenesis (Gooding *et al.*, 2004, Bierie *et al.*, 2003, Marchenko *et al.*, 2002, Polakis, 2000).

Several studies have addressed the effect of *H. pylori* infection in β-catenin signaling and have shown that *H. pylori* leads to the delocalization of β-catenin from the cell membrane (Bebb *et al.*, 2006, Weydig *et al.*, 2007, Suzuki *et al.*, 2005, Franco *et al.*, 2005, Murata-Kamiya *et al.*, 2007, Franco *et al.*, 2008, Sokolova *et al.*, 2008, Suzuki *et al.*, 2009, Nakayama *et al.*, 2009, Hung *et al.*, 2009, Gnad *et al.*, 2010, Kurashima *et al.*, 2008). *H. pylori* phosphorylates and inactivates GSK3β (Tabassam *et al.*, 2009, Sokolova *et al.*, 2008, Nakayama *et al.*, 2009, Suzuki *et al.*, 2009) and, by suppressing GSK3β activity *H. pylori* leads to inhibition of β-catenin phosphorylation and ubiquitin-dependent degradation (Sokolova *et al.*, 2008). *H. pylori*-mediated GSK3β suppression occurs through activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K) / (Akt) signaling pathway *via* EGFR and c-Met receptor (Nagy *et al.*, 2011, Tabassam *et al.*, 2009, Sokolova *et al.*, 2008, Yan *et al.*, 2009, Nakayama *et al.*, 2009, Suzuki *et al.*, 2009).

In one report however, β-catenin stabilization was described to occur in a phosphorylation-independent process (Franco *et al.*, 2005).

The canonical Wnt-signaling pathway appears to be involved in the regulation of β-catenin in the context of *H. pylori* infection. In fact, it has been shown that after *H. pylori* infection there is increased synthesis of Wnt10A by the epithelial cells (Kirikoshi *et al.*, 2001). Additionally, *H. pylori*-induced activation of β-catenin was shown to involve the phosphorylation of the Wnt pathway co-receptor low density lipoprotein receptor-related protein LRP6 and proteins of the dishevelled family, namely Dvl2 and Dvl3 (Gnad *et al.*, 2010).

Several studies report β-catenin translocation to the nucleus after *H. pylori* infection (Suzuki *et al.*, 2005, Franco *et al.*, 2005, Murata-Kamiya *et al.*, 2007, Franco *et al.*, 2008, Sokolova *et al.*, 2008, Suzuki *et al.*, 2009, Nakayama *et al.*, 2009, Hung *et al.*, 2009, Gnad *et al.*, 2010). There are however two studies in non-gastric cell lines that did not show nuclear translocation or activation of β-catenin signaling after infection (Bebb *et al.*, 2006, Ogden *et al.*, 2008). β-catenin nuclear translocation induced by *H. pylori* leads to TCF/LEF-dependent transcription of genes involved in carcinogenesis, including cyclin D1 (Murata-Kamiya *et al.*, 2007, Sokolova *et al.*, 2008, Nakayama *et al.*, 2009, Gnad *et al.*, 2010, Kurashima *et al.*, 2008). This effect was shown to be CagA-dependent in the majority of studies (Suzuki *et al.*, 2005, Franco *et al.*, 2005, Murata-Kamiya *et al.*, 2007, Suzuki *et al.*, 2009, Hung *et al.*, 2009, Kurashima *et al.*, 2008), with the exception of one study performed in a non-human, non-gastric cell line (Sokolova *et al.*, 2008). Additional effectors of the T4SS, like peptidoglycan (Nagy *et al.*, 2011, Viala *et al.*, 2004), and other *H. pylori* virulence factors, such as the multifunctional toxin VacA (Nakayama *et al.*, 2009, Cover *et al.*, 2005) and the outer membrane protein OipA (Franco *et al.*, 2008, Yamaoka, 2011), may also be involved. The use of the Mongolian gerbil model has shown that *H. pylori* infection induces gastric carcinoma precursor lesions, with aberrant β-catenin expression, that led to transcriptional up-regulation of genes implicated in carcinogenesis. In this model-system, and in agreement with most of the cell-line models, aberrant β-catenin expression was associated with infection with *H. pylori* CagA-positive strains (Franco *et al.*, 2005, Franco *et al.*, 2008).

H. pylori and p120-catenin

p120-catenin (p120) is another important component in adherens junctional complex stability and signaling (Hartsock *et al.*, 2008). p120 binds to the juxtamembrane domain of E-cadherin (Yap *et al.*, 1998), and mutations in this domain abolish E-cadherin-mediated

cell-cell adhesion (Kaurah *et al.*, 2007). In addition to E-cadherin stabilization, p120 can also interact with the transcription factor Kaiso (Daniel *et al.*, 1999, van Hengel *et al.*, 1999, Roczniak-Ferguson *et al.*, 2003).

Two reports show that *H. pylori* alters the cellular localization and the phosphorylation status of p120, but not total p120 protein levels (Krueger *et al.*, 2007, Ogden *et al.*, 2008). Krueger *et al.* showed that after six hours of infection of primary gastric epithelial cells, there was a recruitment of the non-phosphorylated p120 to perinuclear vesicles, whereas the fraction of phosphorylated p120 increased and could be detected in the nucleus, at the cell membrane, and at the leading edge of migrating cells (Krueger *et al.*, 2007). Those alterations were associated with elongation of cells and increased migration. Ogden *et al.* also showed nuclear translocation of p120 after *H. pylori* infection of MKN28 cells and of *ex vivo* mouse gastric glands. Nuclear translocation of p120 induced by *H. pylori* was associated with increased MMP-7 mRNA, and occurred by release of the MMP-7 transcriptional repressor Kaiso. These changes were associated with reduced levels of p120 tyrosine phosphorylation, while total p120 remained unchanged (Ogden *et al.*, 2008). *H. pylori*-mediated p120 nuclear translocation was dependent on factors of the T4SS, but independent of the T4SS effector CagA (Ogden *et al.*, 2008).

2. *H. pylori* infection and host epithelial cell signaling

Due to its close interaction with host cells, *H. pylori* has the capacity to induce alterations in the normal host cell signaling pathways. The alterations in the normal cell signaling by *H. pylori* can result in the induction of apoptosis, morphological changes, like the establishment of the “hummingbird phenotype,” disruption of the epithelial barrier, alteration in cell proliferation, and production of pro-inflammatory cytokines (Chen *et al.*, 1997, Segal *et al.*, 1999, Amieva *et al.*, 2003, Fan *et al.*, 1996, Crabtree *et al.*, 1995)

As a result of its localization at the cell membrane receptors tyrosine kinase (RTKs) are among of the first host cell molecules to be in contact with *H. pylori* during infection.

The RTKs are transmembrane proteins that, typically, after activation by their ligands, dimerize or oligomerize (Lemmon *et al.*, 1994, Lemmon *et al.*, 1998, Hubbard *et al.*, 2000, Schlessinger, 2000), making possible an efficient cross-phosphorylation of their adjacent cytoplasmic tyrosine kinase domains. The phosphorylation of the tyrosine residues, a characteristic of the transmembrane signaling, creates docking sites for several molecules involved in a plethora of signal transduction pathways.

Overexpression and mutations of the RTKs signaling pathways elements are associated with carcinogenesis and are targets for anticancer therapy (Brunelleschi *et al.*, 2002).
RTKs such as EGFR and c-Met are basolateral localized in polarized cells (Crepaldi *et al.*, 1994, Maratos-Flier *et al.*, 1987). After the disruption of cellular junctions mediated by *H. pylori*, the polarity of host cell is altered and the RTKs may be now localized apically, in a cellular localization more accessible to the bacteria. Because of these facts, the study of the *H. pylori*-mediated alterations in host cell signaling is critical to understand the outcomes of the infection.

2.1. c-Met signaling pathway

The c-Met receptor is initially synthesized as a 170-kDa single chain intracellular precursor that after processing yields a mature 190 kDa disulfide-linked heterodimer form, which is cell surface-associated. The heterodimer is constituted of a 50 kDa α-chain, entirely extracellular, and a 140 kDa β-chain, that spans the membrane, and consists of an extracellular domain, a membrane spanning domain, and a cytoplasmic tyrosine kinase domain (Giordano *et al.*, 1989) (Figure 6).
In a non-pathological context, c-Met activation by its natural ligand, the hepatocyte growth factor (HGF) also known as scatter factor (SF), occurs in a wide range of tissues, and is required for embryonic development (Uehara *et al.*, 1995, Borowiak *et al.*, 2004), liver regeneration (Borowiak *et al.*, 2004, Huh *et al.*, 2004), and wound healing (Chmielowiec *et al.*, 2007). These cellular processes involve cytoskeleton reorganization, cell motility, proliferation, morphogenesis, invasion, and angiogenesis (Birchmeier *et al.*, 2003, Jiang *et al.*, 2005, Peruzzi *et al.*, 2006).
In a pathological context, abnormal c-Met signaling contributes to tumorigenesis, particularly to the invasive and metastatic phenotype (Furge *et al.*, 2000). Deregulation of c-Met signaling by mutations, overexpression, and autocrine and paracrine activation, is associated with the severity of human cancers, including gastric carcinoma (Kuniyasu *et al.*, 1992, Kaji *et al.*, 1996, Amemiya *et al.*, 2000, Birchmeier *et al.*, 2003). It is known that the disruption of the c-Met multifunctional docking site impairs oncogenic transformation and invasive growth of tumor cells (Bardelli *et al.*, 1998). In addition, c-Met phosphorylation stimulates epithelial to mesenchymal transition (EMT), a key feature of metastization (Birchmeier *et al.*, 2003, Benvenuti *et al.*, 2007).

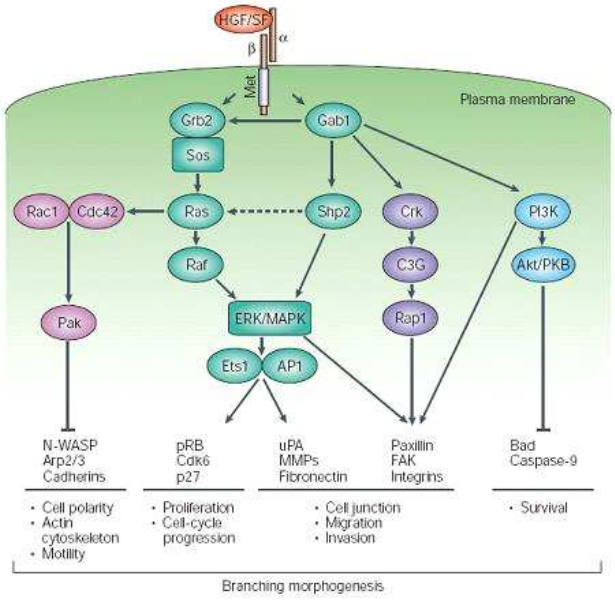


Figure 6. Activation of c-Met results in the recruitment of adaptor molecules that activate signaling pathways resulting in different cellular phenotypes. *Adapted by permission from Nature Reviews in Molecular Cell Biology (Birchmeier et al.) © 2003.*

Regarding the association of *H. pylori* infection with c-Met, studies in which human biopsies were analyzed, showed that an increase in c-Met and HGF expression was associated with the presence of the infection, both in pre-neoplasic and in neoplasic lesions (Hori *et al.*, 2000, Zhuang *et al.*, 2001). However, there are also studies indicating that no correlation exists between c-Met expression and *H. pylori* infection in gastric carcinoma (Kubicka *et al.*, 2002).

It was demonstrated that *H. pylori* infection triggers HGF-independent activation of the c-Met receptor. After injection, CagA can directly bind to the intracellular part of the receptor, functioning as an adaptor protein (Churin *et al.*, 2003) and leading to the activation of the downstream targets PLC-γ and ERK (Churin *et al.*, 2003, Franke *et al.*, 2008). As a result of the activation of these pathways, *H. pylori* induces a motogenic response in host cells (Churin *et al.*, 2003). By mimicking the action of HGF, *H. pylori* is able to modulate the host cell function, thus contributing to the establishment of an invasive phenotype.

It has also been shown that *H. pylori* is able to induce the interaction of the lipid raft-associated transmembrane adaptor proteins NTAL and LIME with c-Met, leading to the activation of a c-Met-Grb2-ERK-cPLA2 signaling cascade at early stages of infection independently of the T4SS (Rieke *et al.*, 2010).

2.2. EGFR signaling pathway

EGFR is a 170 kDa transmembrane protein with a ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (Carpenter, 1987). This receptor is part of a family of human epidermal growth factors receptors, composed of four members: HER1/erbB1 (EGFR), HER2/erbB2, HER3/erbB3, and HER4/erbB4). In contrast with the c-Met receptor, several natural ligands of EGFR are known: EGF, amphiregulin, TGF-α, betacellulin, heparin binding EGF (HB-EGF), and epiregulin (Yarden, 2001). Upon ligand binding, EGFR homo or heterodimerizes, causing autophosphorylation of the tyrosine kinase domain and activation of downstream signal transduction pathways. EGFR can also be transactivated by G-protein-coupled receptors (GPCRs) and adhesion molecules (Prenzel *et al.*, 1999, Pece *et al.*, 2000).

In a non-pathological context, EGFR that is expressed in several cell types, is involved in normal cell proliferation, differentiation, growth, cell death, motility, and matrix adhesion (Yarden *et al.*, 2001).

In a pathological context, EGFR, which is codified by the pro-oncongene *c-erb*^{B}, can be deregulated either by overexpression or mutations, leading to activation of the PI3K, the Ras-Raf-MAPK, and the PLC pathways (Gusterson *et al.*, 1984, Wong *et al.*, 1992, van der Geer *et al.*, 1994, Moscatello *et al.*, 1995, Lynch *et al.*, 2004), a set of alterations associated with multiple cancer types (Normanno *et al.*, 2006). In gastric carcinoma, EGFR is commonly overexpressed, and amplifications of the gene are associated with poor prognosis (Zheng *et al.*, 2004). In the stomach, the signaling pathway triggered by EGF is associated with inhibition of gastric acid secretion and with impaired wound healing (Konturek *et al.*, 1990, Shaw *et al.*, 1987).

Regarding the role of EGFR in cancer, it is also known that the treatment with EGFR monoclonal antibodies or the stable transfection of EGFR antisense results in growth arrest of gastric cell lines or tumors of xenografted mice when EGFR overexpression exists (Teramoto *et al.*, 1996, Hirao *et al.*, 1999).

Concerning the connection between EGFR and *H. pylori,* there are several reports indicating that the infection is associated with an increase in the EGFR and EGF levels, which return to normal after bacteria eradication (Coyle *et al.*, 1999, Wong *et al.*, 2001, Ashktorab *et al.*, 2007). In the same line, the bacteria itself is able to induce an increase in EGFR mRNA levels (Ashktorab *et al.*, 2007, Keates *et al.*, 2007).

It is described that *H. pylori* infection, or purified VacA, are able to activate the EGFR (Wallasch *et al.*, 2002, Caputo *et al.*, 2003). EGFR activation can be achieved, in a T4SS-independent manner, by the HB-EGF up-regulation and release through the action

of proteases (Wallasch *et al.*, 2002), or by transactivaction of TLR4 (Basu *et al.*, 2008). In a process similar to a feedback loop, it has also been described that *H. pylori*-induced IL-8 is involved in the mechanism of processing of EGFR ligands and in EGFR activation (Joh *et al.*, 2005, Beswick *et al.*, 2008).

To prolong the action of the EGFR-activated receptor, *H. pylori* is also able to block its endocytosis, and receptor degradation, in a process dependent on CagA and on the tyrosine kinase c-Abl that is able to phosphorylate the receptor (Bauer *et al.*, 2009) (Figure 7).

The EGFR activation after infection with *H. pylori* results in the activation of PI3K, Akt, Raf, MEK and ERK (Du *et al.*, 2007, Tabassam *et al.*, 2009, Wiedemann *et al.*, 2012) (Figure 7). However, is it also described that VacA is able to inactivate EGFR, and consequently ERK, inhibiting cell scattering and elongation (Tegtmeyer *et al.*, 2009).

H. pylori-mediated activation of EGFR results in several cellular alterations, stressing the involvement of different downstream pathways. The EGFR activation is reported to be involved in enhancement of IL-8 production (Wallasch *et al.*, 2002), and in up-regulation of VEGF, through the action of the *H. pylori* protein HP0175 (Caputo *et al.*, 2003, Basu *et al.*, 2008). EGFR activation by the bacteria also leads to induction of Egr-1 (Keates *et al.*, 2005), activation of human beta-defensin 3 (hBD3) (Boughan *et al.*, 2006), and paxillin phosphorylation (Tabassam *et al.*, 2011). *H. pylori*-induced EGFR activation results in alteration of cytoskeleton and motility, as well as in an anti-apoptotic mechanism, involving Akt and Bcl family members (Yan *et al.,* 2009), and in gastrin induction (Wiedemann *et al.*, 2012).

Overall, these results suggest that the signaling pathways activated by EGFR influenced by *H. pylori* may result in increased risk for neoplasic transformation of epithelial cells.

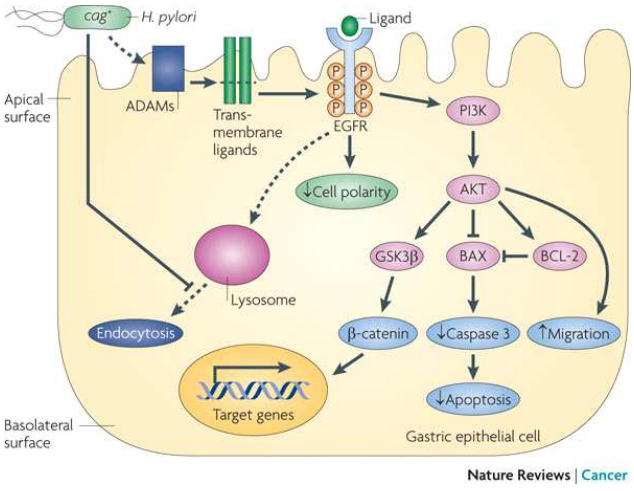


Figure 7. Transactivation of EGFR by *H. pylori* and host cellular induced alterations. *Adapted by permission from Nature Reviews in Cancer (Polk and Peek) © 2010.*

3. Metalloproteinases

Metalloproteinases (MMPs) are a family of zinc dependent enzymes that share a catalytic and a pro-domain, and possess the ability to degrade all the components of the extracellular matrix. MMPs are divided in subfamilies: collagenases, gelatinases, stromelysins and membrane-type MMPs, depending on their structure and subtract (Chakraborti *et al.*, 2003) (Figure 8). After transcription, the majority of MMPs are secreted in an inactive form, a pro-enzyme (zymogen) that has to be processed to become active, both autocatalytically and by other proteinases (Chakraborti *et al.*, 2003, Visse *et al.*, 2003).

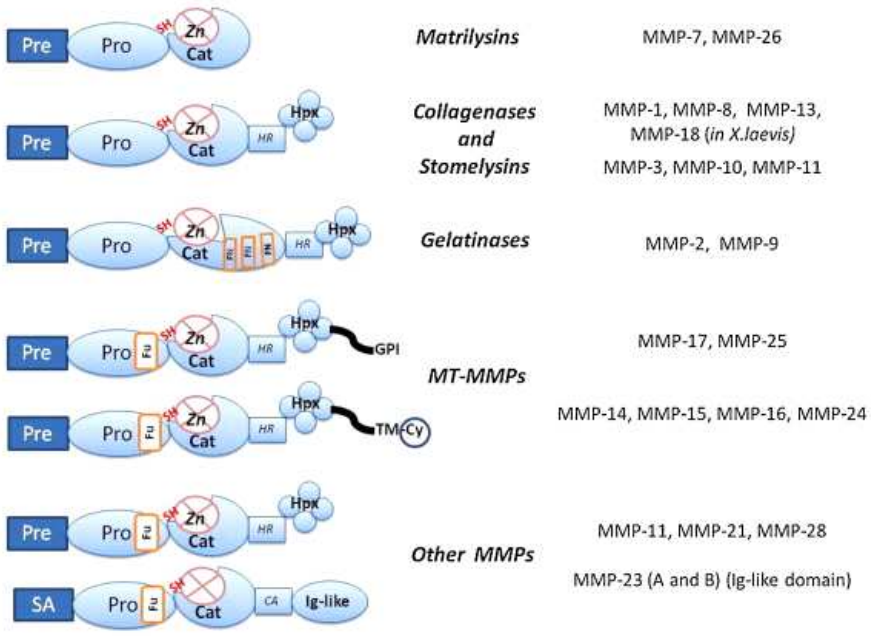


Figure 8. Members of the matrix metalloproteinase family of degrading enzymes. *Adapted by permission from Progress in Histochemistry and Cytochemistry (Mannello and Medda) © 2012.*

Latent MMPs contain a conserved cysteine residue in the pro-enzyme domain with the ability to bind the catalytic zinc. Whenever this bound exists, the enzymatic activity of the MMP is impaired. The physical activation occurs when this binding is disrupted, in a

process named “cysteine switch,” which can occur by physical and chemical means, for example by the action of mercuric compounds, reactive oxygen species or even other MMPs (Chakraborti *et al.*, 2003) (Figure 9).

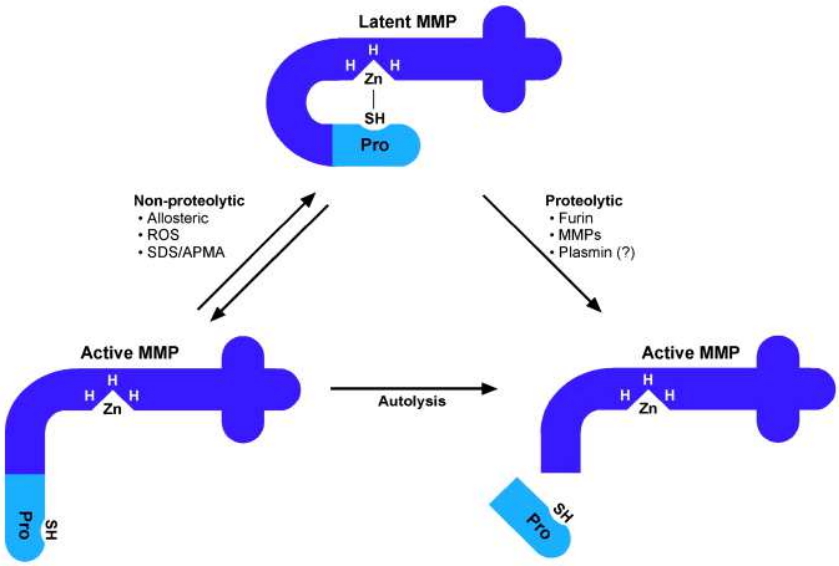


Figure 9. Mechanism of pro-MMP activation**.** Latent MMP is catalytic inactive due to the interaction between the cysteine of the pro-domain and zinc ion of the catalytic domain. Proteolytic cleavage of the pro-domain leads to MMP activation. *Adapted by permission from Matrix Biology (Ra and Parks) © 2007.*

MMPs are tightly regulated at several levels: transcription, compartimentalization, pro-enzyme activation and inactivation, and by the action of specific inhibitors, the tissues inhibitors of MMPs (TIMPs) (Parks *et al.*, 2004).

The main function of MMPs is extracellular matrix degradation, but they are also responsible for the release of growth factors, the modification of cell surface receptors, and the regulation of inflammation and immunity (McCawley *et al.*, 2001, Egeblad *et al.*, 2002, Parks *et al.*, 2004). MMPs are involved in physiological processes such as uterine involution, and in pathological conditions such as tissue damage in inflammatory disease such as arthritis, and cancer invasion (Chakraborti *et al.*, 2003).

Invasion is a complex cell phenotype that implicates various cellular activities that mobilizes multiple molecular pathways within the cell, in which MMPs play an important role. Cell invasion occurs both in non-pathological conditions such as embryonic development and normal immune response, and in pathological conditions such as cancer (Monteagudo *et al.*, 1990, Takeichi, 1991, Pyke *et al.*, 1992, Brooks *et al.*, 1996), in tightly regulated processes. For invasion to occur, cells have to undergo several changes in cell-cell and cell-matrix adhesion, migration, proteolysis, and ectopic survival.

The loss of cell-cell adhesion is essential for invasion and involves alteration in the components of adherens junctions, tight junctions, and desmossomes. One of the key molecules in this process is E-cadherin, that can be downregulated either transcriptionally, by mutations and by promoter methylation, and post-transcryptionally by phosphorylation, glycosilation, proteolysis, endocytosis and sterical hindrance (Vleminckx *et al.*, 1991, Boterberg *et al.*, 2000, Noe *et al.*, 2001, Piedra *et al.*, 2001, Zhu *et al.*, 2001, Machado *et al.*, 2001a).

For cell migration, usually described as formation of stress fibers, membrane ruffles, lamellipodia and filopodia (Nobes *et al.*, 1999), the cell-matrix adhesion has to be altered. Cell-matrix bounds have to be broken, and new transient cell-matrix connections that allow the cell to move are established (Sheetz *et al.*, 1999). Key components of this process are the integrin family of receptors and the components of the focal adhesion complexes formed when a new cell-matrix bound is established, such as FAK, c-Src, and vinculin.

For invasion, cells also need to overcome the matrix, which is constituted by laminins, collagens and proteoglycans. This process occurs through a tightly regulated proteolysis mechanism, in which MMPs have a pivotal role. MMPs are able to degrade all components of the extracellular matrix, eliminating the physical barrier to the cell movement, and creating the route to the migrating cells. Furthermore, MMPs are important because of their involvement in the release and activation of latent proteases, growth factors, and chemotactic agents. Through the action of MMPs, new integrin binding sites on ECM can become exposed and available to transmit migratory and survival signals. At the same time, MMPs are able to cleave cell surface receptor molecules, such as E-cadherin, contributing to the invasive potential of the cells, or promote the ectodomain shedding of transmembrane receptors with pro-invasive properties (Mareel *et al.*, 2003, Noe *et al.*, 1999, Ryniers *et al.*, 2002).

After reaching a new environment, the invasive cells need to remain viable, a condition achieved through the stimulating of anti-apoptotic signals (Raff, 1992). Curiously, many of the antiapoptotic signals are the same signals that drive migration (Mareel *et al.*, 2003).

In the above mentioned mechanisms, several molecules are involved such as c-Met, c-Src, Rho GTPases, PI3K, members of the ERK/MAPK signaling and calpain, which have also been associated with tumors with high invasive potential (Carragher *et al.*, 2004).

Therefore, the cell invasive phenotype is the result of multiple molecular pathways that branch and interact with each other, establishing a complex but also flexible intracellular system.

3.1. *H. pylori* infection and MMPs

H. pylori infection up-regulates the expression and activity of several MMPs both *in vitro* and *in vivo*. Although the molecular mechanisms that lead to MMP modulation are not well established, the role of MMPs in *H. pylori*-mediated cell invasion, migration and processing of growth factors has been reported (Wallasch *et al.*, 2002, Wroblewski *et al.*, 2003, Wu *et al.*, 2005, McCaig *et al.*, 2006, Oliveira *et al.*, 2006, Yin *et al.*, 2010).

The majority of the data associating MMPs up-regulation and *H. pylori* infection came from analysis of cDNA arrays that compared infected versus non-infected conditions. These studies showed that *H. pylori* is associated with upregulation of all MMP subfamilies, namely MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13, MMP-14, MMP-15, and MMP-19 (Yokoyama *et al.*, 2000, Guillemin *et al.*, 2002, Gooz *et al.*, 2003, Kitadai *et al.*, 2003, Mori *et al.*, 2003, Wroblewski *et al.*, 2003, Krueger *et al.*, 2006). In addition, *H. pylori* itself is able to secrete a MMP-3 like enzyme (Windle *et al.*, 1997, Gooz *et al.*, 2001). Modulation of MMPs by *H. pylori* is best documented for MMP-1, MMP-7 and the gelatinases MMP-2 and MMP-9.

It is reported that *H. pylori* up-regulate MMP-1 both in fibroblasts (Yokoyama *et al.*, 2000) and in epithelial cells from infected individuals with gastritis and gastric cancer (Krueger *et al.*, 2006, Wu *et al.*, 2006, Pillinger *et al.*, 2007). MMP-1 expression is dependent on the T4SS, OipA (Krueger *et al.*, 2006, Wu *et al.*, 2006), or CagA (Pillinger *et al.*, 2007), depending on the cell line and strains used in different studies.

MMP-1 up-regulation occurs in an ERK and JNK-dependent manner (Krueger *et al.*, 2006, Pillinger *et al.*, 2007), through activation of the Ras/Raf/RhoA/MAPK pathway (Wu *et al.,* 2006), and under the regulation of PKC (Sokolova *et al.*, 2012). The increase in MMP-1 in the context of *H. pylori* infection has been associated with increased basement membrane degradation and cell invasion (Krueger *et al.*, 2006, Sokolova *et al.*, 2012).

H. pylori-mediated MMP-7 expression has been reported and the bacterial virulence factors VacA, T4SS, CagA, and OipA may play a role in this process (Bebb *et al.*, 2003, Crawford *et al.*, 2003, Wroblewski *et al.*, 2003, Koyama, 2004, Yanagisawa *et al.*, 2005, Chung *et al.*, 2010, Eftang *et al.*, 2012, Wu *et al.*, 2006, Ogden *et al.*, 2008).

In vitro, MMP-7 is expressed in epithelial cells at the advancing edge of migrating cells (Wroblewski *et al.*, 2003) and *in vivo* at the epithelial cells surface, and in the proliferative zone of the gastric glands (Wroblewski *et al.*, 2003, Koyama, 2004, Bebb *et al.*, 2003), and is also expressed in lymphocytes (Koyama, 2004).

The signaling pathways underlying the *H. pylori*-mediated in MMP-7 expression are also controversial. *H. pylori*-associated cytokines IL-8, TNF-α and IL-6, as well as EGFR

ligands, may have a role in the process due to their ability to induce MMP-7 expression, and a role for gastrin has also been suggested (Wroblewski *et al.*, 2003). MMP-7 expression occurs in a pathway involving Rho, Rac and the NF-kB (Wroblewski *et al.*, 2003), in a ERK-dependent manner (Crawford *et al.*, 2003), or through aberrant p120 catenin activation, that after nuclear translocation relieves the MMP-7 repressor Kaiso (Ogden *et al.*, 2008).

MMP-7 is involved in *H. pylori*-induced migration and invasion (Wroblewski *et al.*, 2003), in the processing of insulin-like growth factor binding protein (IGFBP)-5 (McCaig *et al.*, 2006) and in HB-EGF shedding (Yin *et al.*, 2010). IGFBP-5 and HB-EGF have a role, respectively, in proliferation and migration of gastric myofibroblast (McCaig *et al.*, 2006), and in EGFR activation and IL-8 production (Yin *et al.*, 2010, Wallasch *et al.*, 2002).

H. pylori infection is associated with increased expression of the MMP-2 and MMP-9 gelatinases (Bergin *et al.*, 2004, Kundu *et al.*, 2006). MMP-2 and MMP-9 are expressed in epithelial cells and lymphocytes and MMP-9 is also expressed in fibroblast and macrophages (Koyama, 2004, Mori *et al.*, 2003, Danese *et al.*, 2004, Bergin *et al.*, 2004). MMP-2 expression is induced by *H. pylori* in a CagA and T4SS-dependent manner (Yanagisawa *et al.*, 2005), and is stimulated by the *H. pylori*-induced IL-21, in a process NF-kB dependent (Caruso *et al.*, 2007).

H. pylori-mediated MMP-9 expression is dependent on different bacterial virulence factors such as T4SS, CagA, OipA, HP-NAP, HpaA, urease, or LPS, depending on the cell line models and bacterial strains used (Yanagisawa *et al.*, 2005, Mori *et al.*, 2003, Wu *et al.*, 2006, Nam *et al.*, 2011, Kundu *et al.*, 2006, Bergin *et al.*, 2005).

H. pylori-induced MMP-9 up-regulation in epithelial cells occurs via NF-kB (Mori *et al.*, 2003, Nam *et al.*, 2011, Wu *et al.*, 2005, Caruso *et al.*, 2007), COX-2 (Wu *et al.*, 2005), CagA phosphorylation, SHP-2 and ERK (Nam *et al.*, 2011). In addition, the infection-associated cytokines TNF-α, IL-1β, IL-6, and IL-21 are able to induce MMP-9 secretion (Caruso *et al.*, 2007, Kundu *et al.*, 2006). Furthermore, *H. pylori* eradication leads to decreased MMP-9 in patients with chronic gastritis and gastric ulcers (Danese *et al.*, 2004, Kubben *et al.*, 2007, Kundu *et al.*, 2011).

H. pylori-induced increase in MMP-2 and MMP-9 activities results in increased cell invasion (Oliveira *et al.*, 2006, Wu *et al.*, 2005).

OUTLINE AND AIMS

The general aim of the work presented in this thesis was to evaluate the role of *H. pylori* infection in the induction of host gastric epithelial cell invasion. The initial approach consisted on the evaluation of the invasive capacity through extracellular matrix components of the non-invasive human gastric cancer cell line AGS after infection with *H. pylori*. The role of the bacterial virulence factors CagA, T4SS, and VacA was also evaluated. After establishing that the c-Met receptor was important for *H. pylori*-mediated AGS cell invasion, the role of the downstream targets of c-Met was also elucidated. Additionally, and after demonstrating that Nck, PLC-γ, and c-Src were involved in *H. pylori*-mediated cell invasion, the activation of these molecules by *H. pylori* was studied. Studies in this section included *in vitro* infections of cell lines with *H. pylori* strains, followed by assessment of cell invasion in Matrigel assays. Studies also included transient transfection of cell lines with siRNAs for gene silencing, as well as co-immunoprecipitation and western blot analyses. Data obtained are presented in Part I.1 of the Results.

Based on the results from Part I.1 pointing to a role of *H. pylori* in inducing extracellular matrix degradation by host cells, and since matrix metalloproteinases are important for that process, the next aim was to evaluate the role of *H. pylori* in MMP expression. Studies started with the validation of a panel of MMPs shown to be up-regulated in a cDNA expression microarray performed by our Group. After confirmation that *H. pylori* increased the expression of MMP-1, MMP-7, and MMP-10, a more detailed investigation of the effect of *H. pylori* on MMP-10 modulation was performed. Quantitative real-time polymerase chain reaction, western blot, and enzymatic activity assays were used in uninfected and *H. pylori*-infected cells. The role of the *H. pylori* virulence factors CagA and T4SS was addressed using bacterial mutants, and was further confirmed using a panel of *H. pylori* clinical isolates with known *cag*A and T4SS status. The involvement of specific host cell signaling pathways and of receptor tyrosine kinases in the modulation of MMP-10 expression by *H. pylori* was evaluated in experiments with siRNA gene silencing and with chemical inhibitors. The role of MMP-10 in *H. pylori*-mediated cell invasion was assessed with Matrigel assays.These results are presented in Part I.2 of the Results and in Paper I.

Since the E-cadherin-catenin complex functions as an invasion suppressor, the next aim was to evaluate whether the complex had a role in *H. pylori*-mediated cell

invasion and signaling. For that, gastric cell lines with intact E-cadherin-catenin complex were used. Also, and for the purpose of comparison with previous data obtained in the AGS background, an AGS cell line with a wild-type E-cadherin was established. For the characterization of the structure and function of the adherens junctions, immunofluorescence, confocal microscopy and aggregation assays were used. The phosphorylation and interactions between proteins of the adherens junctions were evaluated by co-immunoprecipitation and western blot. The invasion capacity of the different cell lines was assessed with Matrigel assays. Data generated in this part of the thesis is presented in Part II of the Results and in Paper II.

In this Thesis, a paper summarizing the findings of the literature regarding *H. pylori* infection and the adherens junctions (Paper III) and a paper reviewing the role of E- and P-cadherins in cancer (Paper IV) are also included, and have been partially used in the Introduction.

MATERIALS AND METHODS

1. Cell cultures

AGS (ATCC CRL-1739), NCI-N87 (ATCC CRL-5822), and IPA220 cells (Gartner *et al.*, 1996), all derived from human gastric carcinomas were maintained in RPMI 1640 (Gibco), with 10% fetal bovine serum (FBS) (Hyclone), 2.5 µg/ml fungizone (Gibco), 200 µg/ml streptomycin, and 200 IU/ml penicillin (Gibco) at 37ºC, under a 5% CO2 humidified atmosphere. The AGSEcad cell line was maintained in the same conditions with supplementation with 5 µg/ml of blasticidin (Invitrogen) for clonal selection.The adherent cells were detached using trypsin (Gibco).

2. *H. pylori* strains and growth conditions

Bacteria were grown in tryptic soy agar (TSA) supplemented with 5% sheep blood (BD Bioscience) and incubated 48 hours at 37ºC under a microaerophilic atmosphere. Experiments were performed with wild-type *H.pylori* strains 60190 (ATCC 49503), 26695 (ATCC 700392), 84183 (ATCC 53726), Tx30a (ATCC 51932), and G27 (Xiang *et al.*, 1995), as well as with the mutant strains 60190Δ*vac*A, 60190Δ*cag*A, 60190Δ*cag*E, 84183Δ*cagA* and 84183Δ*cagE* (all mutant strains were kindly provided by Professor John Atherton, Nottingham University, UK). A panel of clinical isolates with known *vac*A, *cag*A and *cag* PAI statuses was also used for some experiments (CI-7, CI-43, CI-45, CI-50, CI-51, CI-59, CI-60, CI-62, CI-63, and CI-69).

Table I: Characterization of *H. pylori* strains in terms of virulence factors *vacA*, *cagA* and *cag* PAI

Bacterial strain	**Virulence factor**		
	vacA	***cagA***	***cag* PAI**
26695	s1/m1	+	+
60190	s1/m1	+	+
60190Δ*cag*A	s1/m1	-	+
60190Δ*cag*E	s1/m1	+	-
60190Δ*vac*A	-	+	+
84183	s1/m1	+	+

84183Δ*cag*A	s1/m1	-	+
60190Δ*cag*E	s1/m1	+	-
CI-7	s2/m2	-	-
CI-43	s1/m1	+	+
CI-45	s2/m2	-	-
CI-50	s1/m1	+	+
CI-51	s2/m2	-	-
CI-59	s1/m1	+	+
CI-60	s1/m1	+	+
CI-62	s1/m1	-	nd
CI-63	s1/m2	-	-
CI-69	s1/m1	+	+
G 27	s1/m1	+	+
Tx30a	s2/m2	-	-

(+: present; - : absent; nd: not determined)

3. Infection of gastric cells

80% confluent monolayers were washed in phosphate-buffered saline (PBS), pH=7.4, and incubated in serum and antibiotic-free medium (Gibco) at least 3 hours before the infection. To perform the infections, the bacteria were collect from the agar plate with PBS, and the bacterial density was estimated by absorbance at 600 nm (OD=0.1 represents $2x10^6$ bacteria/μl). *H. pylori* were added to the host cells at a multiplicity of infection (MOI) of 100. Cultures were maintained at 37ºC under a 5% CO2 atmosphere. After the infection periods, and depending on the specific experiment, conditioned media were collected, and the cells were lysed or used to extract RNA.

4. Invasion assay

The cell invasive phenotype was assessed using 24-well Matrigel-coated invasion inserts of 8 μm pore-size filters (BD Biosciences). Inserts were incubated 1 hour at 37ºC with antibiotic-free medium. After re-hydration, $5x10^4$ cells ressuspended in a final volume of

500µL of medium with 10% FBS and without antibiotics were incubated 24 hours at 37ºC, on top of the filters, in the presence or absence of *H. pylori*. After incubation, filters were washed with PBS and fixed in 4% paraformaldehyde. Non-invasive cells were removed with a cotton swab at the top of the chamber, and invasive cells in the bottom part were stained and mounted in Vectashield with DAPI (Vector Laboratories). The number of invasive cells was assessed by measuring the number of nuclei in the bottom part of the filter, in at least 25 microscopic fields (20X objective), using a Zeiss Imager Z1 fluorescence microscope.

5. Small interference RNA (siRNA) transfection

For siRNA transfection, cells seeded in 6-well plates at 50% confluence were washed with PBS, pH 7.4, and incubated in serum- and antibiotic-free medium. siRNAs targeting c-Src, c-Met, Fer, MMP-10, Nck, PLC-γ and Non-silencing siRNA were obtained from Qiagen, and all the others from the siRNA platform of Max-Planck Institute for Infection Biology, and prepared according to the manufacturer's instructions. Transient transfection of AGS cells with siRNA was performed using Lipofectamine 2000 (Invitrogen) as transfection reagent. All siRNAs were used at a concentration of 100 nM, with the exception of c-Met and PLC-γ at 80 nM, and Nck at 50 nM. The efficiency of siRNA was evaluated 48 hours after transfection by western blot for all the siRNAs used, except for MMP-10, which was evaluated by qRT-PCR.

6. Cell treatment with growth factors, cytokines, and chemical inhibitors

Pharmacological inhibitors U-0126, SB203580, SP600125, and AG-1478 were obtained from Cayman Chemicals, PP2 from Calbiochem, and NK4 was kindly provided by W. Jiang (Department of Surgery, University of Wales College of Medicine, Cardiff UK). The concentrations of inhibitors used were 25 µM for U-0126, 20 µM for SB203580 and SP600125, 5µM for PP2 and AG-1478, and 100 ng/mL for NK4. The cells were treated with the inhibitors 1 hour before the infection, and the inhibitors were not removed from the medium during the experiments.
Recombinant IL-1β and TGF-β (Sigma), IL-10 (Immunotools), and TNF-α (PeproTech) were used at a final concentration of 10 ng/mL, IL-8 at 8 ng/mL, and INF-γ at 100U/mL.

HGF (Immunotools) at 250 ng/mL and EGF (Sigma) at 50 ng/mL were used to treat the cells for 24 hours.
Control monolayers were processed similarly but substituting those compounds by an equivalent volume of PBS or DMSO (in the case of the chemical inhibitors).

7. Preparation of cell lysates and co-immunoprecipitation

At the end of infection period, cells were lysed using cold Catenin lysis buffer (1% Triton X-100, 1% NP-40 in PBS, pH 7.4) with proteases and phosphatases inhibitors (3mM sodium vanadate, 20mM NaF, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10μg/ml leupeptin). Cells were scraped with cold lysis buffer on ice, centrifuged at 14000 rpm for 10 minutes at 4ºC, and soluble proteins were quantified using the Bio-Rad DC™ Protein Assay (Bio-Rad). For analysis of total lysates, 25 μg of protein were used. For co-immunoprecipitation 750 μg of proteins were incubated overnight at 4ºC with mouse monoclonal anti-phosphotyrosine (PY-20), anti-E-cadherin, anti-p120-catenin, anti-c-Met antibodies, or, as a negative control, with an IgG from the same species of the immunoprecipitation antibody. Immunoprecipitated complexes were incubated 1 hour with protein G-sepharose beads (GE Healthcare) washed and pre-incubated in 1% BSA solution. Proteins were separated by reducing 7.5 or 10% SDS-PAGE electrophoresis. The Precision Plus Protein™ Standard Dual Color protein marker (Bio-Rad) was used to identify the molecular weights.

8. Immunoblot analysis

Proteins were transferred onto Hybond nitrocellulose membranes (GE Healthcare), stained with Ponceau S (Sigma), and blocked 30 min with 5% non-fat milk in PBS +0.1% Twen-20 (PBS-T) and incubated overnight at 4ºC with primary antibody, in 5% non-fat milk or 4% BSA (phospho antibodies) in PBS-T. Unbound antibody was washed four times with PBS-T. The membranes were incubated with secondary antibody, in PBS-T with 5% non-fat milk for 45 min at room temperature and washed six times with PBS-T, and then detected using Luminata™ Forte Western HRP Substract (Millipore). For total lysates, equal loading control was determined by reprobing the same membrane with a mouse anti-α-tubulin antibody (Sigma). The ECL membrane images were quantified

using the Image J tool. Immunoblots are representative replicates from at least three experiments.

9. Antibodies

Table 2 presents the information relative to the antibodies used for western blot, co-immunoprecipitation, immunofluorescence, immunohistochemistry, and functional inhibitory assays.

Table 2: Antibodies used during experimental work

Target molecule	Serum	MW (KDa)	Dilutions			IP	Functional assay	Supplier
			WB	IF	IHC			
Primary antibodies								
c-Cbl	mouse	120	1:1000	-	-	-	-	BD Bioscience
c-Met	rabbit	145	1:1000	-	-	-	-	Santa Cruz
c-Met	mouse	-	-	-	-	+	-	Upstate
CagA	rabbit	120	1:500	1:50	-	-	-	Santa Cruz
E-cadherin	mouse	120	1:1000	1:100	-	+		BD Bioscience
E-cadherin (MB2)	Hybrido-ma	-	-	-	-	-	Slow aggrega-tion	Gift (Mark Bracke)
F-actin (Phalloidin -FITC)	-	-	-	1:500	-	-	-	Sigma
FAK	mouse	125	1:1000	-	-	-	-	BD Bioscience
Fer	rabbit	94	1:500	-	-	-	-	Santa Cruz
Gab-1	rabbit	110	1:500	-	-	-	-	Santa Cruz
MMP-1	rabbit	54 / 42	1:1000	-	-	-	-	Millipore
MMP-2	rabbit	72 / 64	1:500	-	-	-	-	Labvision Neomarkers
MMP-9	mouse	92 / 86	1:500	-	-	-	-	Calbiochem
MMP-10	goat	54 / 44	1:2000	-	-	-	-	Santa Cruz

MMP-10	mouse	-	-	-	1:500		-	Abcam
Nck	mouse	48	1:1000	-	-	-	-	BD Bioscience
PLC-γ	mouse	155	1:1000	-	-	-	-	BD Bioscience
PY-20	mouse	-	-	-	-	+	-	BD Bioscience
p120 catenin	mouse	120	1:2000	1:250	-	+	-	BD Bioscience
Shc	mouse	66/52/46	1:1000	-	-	-	-	BD Bioscience
Shp-2	rabbit	72	1:500	-	-	-	-	Santa Cruz
Src	rabbit	60	1:1000	-	-	-	-	Cell Signaling
P-Tyr416-Src	rabbit	60	1:1000	-	-	-	-	Cell Signaling
α-catenin	rabbit	100	1:2500	1:500	-	-	-	Sigma
α-tubulin	mouse	52	1:10000	-	-	-	-	Sigma
β-catenin	rabbit	92	1:2500	1:500	-	-	-	Sigma
Secondary antibodies								
Goat IgG (HRP)	donkey	-	1:2000	-	-	-	-	Santa Cruz
Mouse IgG (HRP)	sheep	-	1:3000	-	-	-	-	GE Healthcare
Rabbit IgG (HRP)	donkey	-	1:5000	-	-	-	-	GE Healthcare
Mouse IgG (Alexa488)	goat	-	1:1000	-	-	-	-	Molecular Probes
Rabbit IgG (Alexa488)	goat	-	1:1000	-	-	-	-	Molecular Probes
Rabbit IgG (Alexa594)	goat	-	1:1000	-	-	-	-	Molecular Probes

(MW: molecular weight; WB: western blot; IF: immunofluorescence; IHC: immunohistochemistry; IP: Immunoprecipitation)

10. Quantification of mRNA by quantitative real-time PCR (qRT-PCR)

The total RNA from the cells was extracted using TriPure Isolation Reagent (Roche). The RNA concentration and purity were determined using a NanoDrop Spectrophotometer ND-1000. 1 µg of RNA was used for cDNA synthesis, using Random Primers and SuperScript® II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using a TaqMan Mix (Applied Biosystems) and the following TaqManprobes (Applied Biosystems): MMP1 (Assay Hs00899658_m1), MMP7 (Assay

Hs01042796_m1) and MMP-10 (Hs00233987_m1). Relative MMP-10 expression was normalized to levels of GAPDH (Human GAPDH Endogenous Control (FAM™ Dye / MGB Probe, Non-Primer Limited), Applied Biosystems). The reactions were carried out with 0.5 µL of cDNA templates in a total volume of 10 µL. Each reaction was performed in triplicate, and the PCR performed in a 7500 Real Time PCR System (Applied Biosystems).

11. Preparation of conditioned media

The conditioned media collected from non-infected and infected cells were centrifuged at 3824 g for 3 min and filtered through 0.2 µm pore size filter (Advantec). To measure the MMP-10 activity, conditioned media were concentrated using a Speed-Vac system. For protein analysis with SDS-PAGE, conditioned media were concentrated using Amicon® ultra-0.5 centrifugal filter devices, 10 kDa cutoff (Millipore), prepared in Laemmli 4x, and 7.5 µL were separated by SDS-PAGE.

12. MMP-10 activity assay

The secreted active MMP-10 present in the conditioned medium, concentrated with the Spedd-Vac system, was measured using the fluorimetric assay SensoLyte® 520 MMP-10 Assay Kit (AnaSpec) according to the manufacturer's instructions. 50µL of each conditioned media sample were used. MMP-10 activity was measured in 96-well microplates. Recombinant MMP-10 (AnaSpec) was used to perform a standard curve of concentrations, and a fluorigenic FAM/QXL™ 520 FRET peptide was used as a substrate for MMP-10. Fluorescence (490 nm excitation and 520 nm emission) was recorded on a fluorimeter Synergy Mx (Biotek).

13. Immunocitochemistry and confocal microscopy

Cell monolayers were grown on glass coverslips, fixed in ice-cold methanol or in 4% paraformaldehyde (actin staining), incubated 30 minutes in 5% BSA and immunostained with anti-E-cadherin, anti-p120-catenin (both BD Biosciences Bioscience), anti-α-catenin, anti-β-catenin (both Sigma), or anti-CagA (Santa Cruz), for 1 hour or with FITC-conjugated phalloidin (Sigma) for 15 minutes. Goat anti-mouse, anti rabbit Alexa488, or

anti-rabbit-Alexa594 conjugated (Molecular Probes) were used as secondary antibodies, and incubated for 45 minutes. Coverslips were washed in PBS and mounted with Vectashield with DAPI (Vector Laboratories). A Leica DMRE2, a Zeiss Imager Z1 fluorescence microscope, or a SP2-SE-AOBS laser-scanning confocal microscope (Leica) was used. Confocal images were deconvolved with Huygens Pro3.2 (SVI, The Netherlands).

14. Transduction of the AGS cell line with human E-cadherin cDNA

Plasmids containing human E-cadherin cDNA were constructed as previously described (Suriano *et al.*, 2003b) and were stably transduced using a pLenti6/V5 expression vector (Invitrogen), according to manufacturer's instructions.
293FT cell line, the lentivirus producer cell line, was maintained in DMEM medium (Gibco) at 37ºC, 5% CO_2. After reaching the appropriate confluence the expression vector plus the ViraPower^{TM} packaging mix were added in order to produce viral particles that were harvested after 24 hours. Posteriorly, these lentivirus were added to AGS cells and maintained for 24 hours. 5 µg/ml of blasticidin were added to the culture medium for clonal selection. Several clones were isolated, using cloning rings and splitting the cells surrounded by them, after which they were then transferred to new cell culture flasks. These clones were then characterized for the expression of E-cadherin by western blot and immunofluorescence.

15. Aggregation assay

AGS and AGSEcad cells were trypsinized, resuspended as single-cells, and $2x10^5$ cells in suspension were added to 96-well agar-coated plates (Bacto Agar). As control for E-cadherin-mediated aggregation, AGSEcad cells were incubated with an anti-E-cadherin antibody (MB2) (kindly provided by Professor Marc Bracke, Ghent University Hospital, Ghent, Belgium). Cells were then incubated for 48 hours at 37ºC in a 5% CO_2 atmosphere. The ability of cells to aggregate or to persist as a single-cell suspension was evaluated microscopically and photographed.

16. Statistical analysis

Data analysis were performed with Student's t-test and expressed as mean values of at least three independent experiments ± standard errors, unless otherwise stated. The comparison between MMP-10 expression and the cagA status of the *H. pylori* clinical isolates was performed using the non-parametric Mann-Whitney test. For that, a variable that represents the average of the assays for each strain was created, and strains were divided into *cag*A-positive and *cag*A-negative groups. Differences were considered significant at P values less than 0.05.

RESULTS

Part I

Part I.1. Analysis of the effects of H. pylori in host gastric epithelial cell invasion

The infection by *H. pylori* induces several well established alterations in the host cells, such as the induction of pro-inflammatory cytokines, enhancement of reactive oxygen and nitrogen species, and structural alterations in the organization of the cytoskeleton associated with increased motility and migration, that are named as "hummingbird phenotype" (Segal *et al.*, 1999, Churin *et al.*, 2001, Naumann *et al.*, 2004). However, one of the less explored phenotypes that may be associated with the bacterial infection is host cell invasion. Invasive cells have the ability to degrade components of the extracellular matrix, usually by an up-regulation of MMPs, and to increase motility, that allows movement through the stromal tissue. The invasive phenotype must be regarded not only as general stromal tissue destruction, but also as a consequence of a complex signaling network that can also result in other cellular processes such as growth factor processing, cytokine release, and signaling transduction.

1.1. Role of *H. pylori* and bacterial virulence factors in host cell invasion

As a first approach to evaluate the effects of *H. pylori* on epithelial cell invasion, AGS cells, derived from a gastric carcinoma and commonly used in *H. pylori* related studies, were used.

Since the presence of virulence factors such as CagA, the *cag* PAI, and the VacA toxin are associated with most severe clinical outcomes related with *H. pylori* infection (Crabtree *et al.*, 1991a, Blaser *et al.*, 1995, Peek *et al.*, 1995, Figueiredo *et al.*, 2002), and since cell invasion may reflect cellular process that ultimately can lead to more severe disease, the role of these virulence factors in cell invasion was also evaluated.

To try to understand this issue, a Matrigel invasion assay was used. In this assay, cells with invasive capacity are able to degrade an artificial matrix, Matrigel, which mimics the extracellular matrix. The cells that overcome that barrier can then be stained and counted. This assay was performed by incubating AGS cells alone, as a control, or with the *H. pylori* strains 26695, 60190, or G27, on Matrigel-coated filters for a period of 24 hours. In comparison with non-infected cells, *H. pylori* infected AGS cells showed a significant increase in the number of invasive cells through the Matrigel-coated filters (Figure 10A).

To study the role of *H. pylori* virulence factors in the invasive phenotype, similar experiments were performed using the wild type strain 60190, and its mutants for *cagA* (60190Δ*cag*A), *cagE* (60190Δ*cagE*), and *vac*A (60190Δ*vac*A). The co-culture of AGS cells with *H. pylori* wild type strain 60190 and the respective mutant strains that lack CagA or have impaired the T4SS (*cag*E mutant) showed that the number of invasive cells in the presence of these mutants was significantly lower than that observed in presence of the wild type strain (Figure 10B). Results also showed that the presence of the T4SS is more critical to the induction of the invasive phenotype than the presence of CagA, indicating that other virulence factors injected by the T4SS may also be involved in the process. The VacA virulence factor is not involved in the induction of the phenotype, since a strain that lacks this virulence factor induces similar levels of invasion compared to the wild type strain.

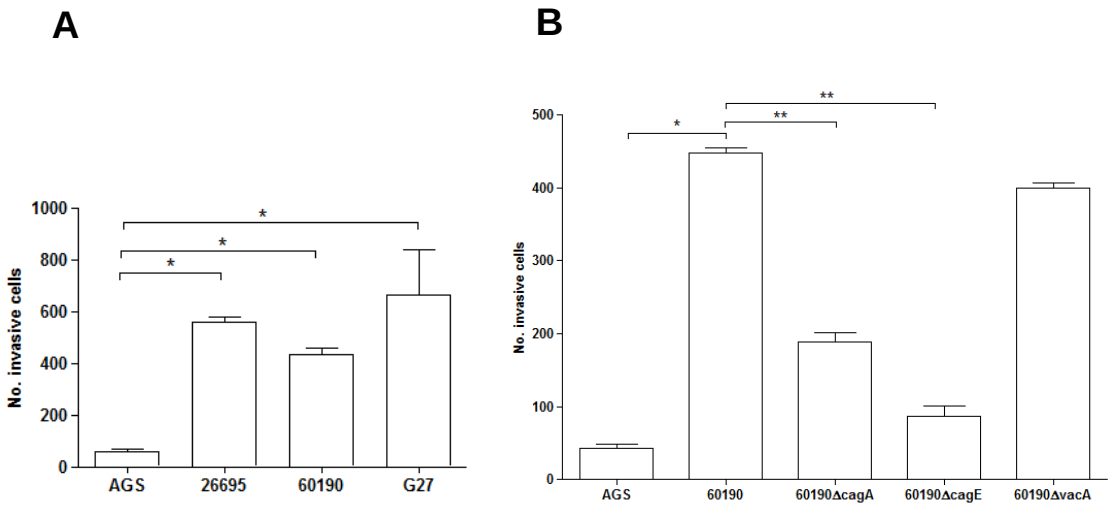


Figure 10 – *H. pylori stimulate AGS cell invasion in a T4SS dependent manner*. Invasion assays on Matrigel coated filters for 24 h. **(A)** AGS cells infected with *H. pylori* strains 26695, 60190, and G27. **(B)** AGS infected with *H. pylori* wild type strain 60190, and its isogenic mutants 60190Δ*cag*A, 60190Δ*cagE* and 60190Δ*vac*A. Data on graph represents the mean value +/- SE and are representative of three independent experiments. *, significantly different from non-infected cells;**, significantly different from cells infected with wild type *H. pylori* strain.

1.2. Role of the c-Met in *H. pylori*-mediated cell invasion

Once established that *H. pylori* is able to induce an invasive phenotype in the host gastric cells, the focus of the research was directed to the molecular mechanisms behind this phenotype. In the cancer context, the RTK c-Met was shown to be a regulator of epithelial

cell invasion (Hasegawa *et al.*, 1995). Furthermore, this receptor was also shown to be activated by *H. pylori* infection leading to alterations in host cell motility (Churin *et al.*, 2003). Therefore, the next experiments were performed to study the involvement of c-Met receptor in *H. pylori*-mediated invasive phenotype. For that, the chemical inhibitor NK4, an antagonist of the natural ligand of c-Met, the hepatocyte growth factor (HGF), as well as a siRNA targeting c-Met were used. AGS cells treated or not with NK4 or siRNA, and infected or not with *H. pylori,* were tested for the invasive capacities in Matrigel-coated filters.

As depicted in the graphs of the Figure 11, both treatments with NK4 and with siRNA directed to c-Met resulted in a significant decrease in the number of invasive cells in the infected conditions. These results clearly point to a role of the c-Met receptor in the induction of invasion mediated by *H. pylori*.

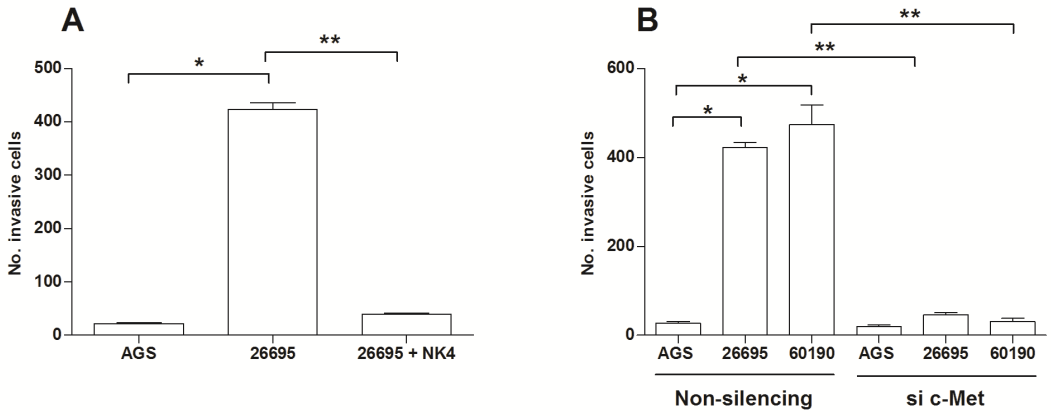


Figure 11 – *c-Met has a role in H. pylori-mediated cell invasion*. Non-treated AGS cells or treated with **(A)** NK4, a chemical inhibitors of c-Met, or **(B)** with a Non-silencing siRNA or an siRNA directed to c-Met, were co-cultured with *H. pylori* for 24 hours at a MOI of 100 on Matrigel coated filters. Data on graph represents the mean value +/- SE and are representative of three independent experiments. *, significantly different from non-infected cells;**, significantly different from cells infected with wild type *H. pylori* strain.

1.3. Role of the c-Met downstream targets in *H. pylori*-mediated cell invasion

Results so far showed that *H. pylori* strains with a functional T4SS induce an increase in host cell invasion through the c-Met receptor. The next step was to identity the signaling molecules downstream c-Met that are implicated in *H. pylori*-mediated cell invasive phenotype.

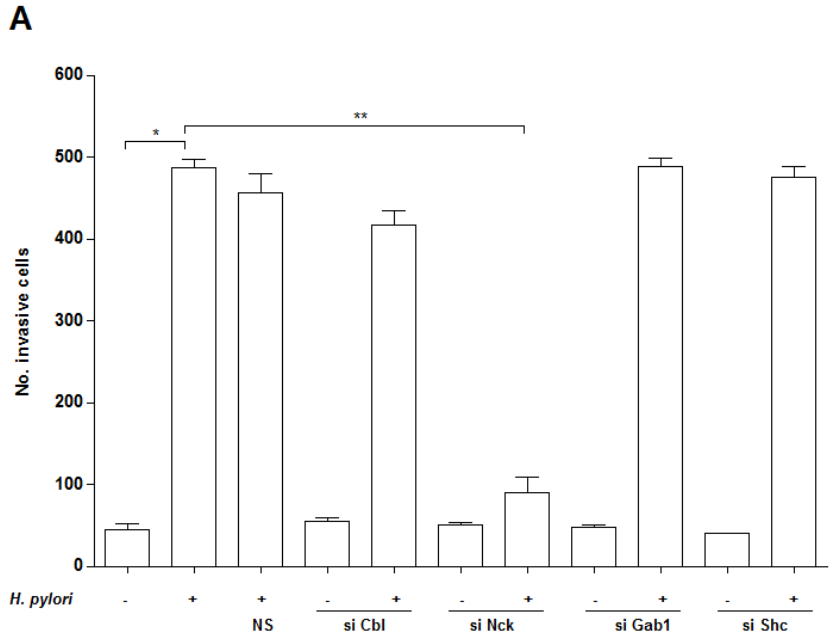
To address this issue a siRNA-based strategy was used. AGS cells were transiently transfected with siRNA abrogating the expression of the c-Met adaptors c-Cbl, Nck, Gab1,

and Shc. Efficient knockdown was achieved and was maximal 48 hours after transfection (Figure 12). Invasion assays were performed 24 hours after transfection, by incubating AGS cells with *H. pylori* strain 26695 on Matrigel filters for an additional 24 hours period.

Only AGS cells transfected with siRNA to Nck showed a significant decrease in the number of invasive cells after *H. pylori* infection when compared with non-silenced control cells (Figure 12A). These findings demonstrate that Nck plays a role in *H. pylori*-mediated cell invasive phenotype.

Using a similar strategy, the involvement of the c-Met downstream targets c-Src, FAK, PLC-γ, and Shp-2 in *H. pylori*-mediated invasion was analyzed. Efficient knockdown was achieved and was maximal 48 hours after transfection (Figure 12C). Only the silencing of c-Src and PLC-γ resulted in a significant decrease in the number of invasive cells in the presence of *H. pylori*, in comparison with non-silenced AGS cells (Figure 12B), showing that c-Src and PLC-γ are involved in *H. pylori*-mediated cell invasion. The silencing of FAK also resulted in a decrease in the number of invasive cells in the presence of the bacteria. However, this difference was not statistically significant (Figure 12B). Nevertheless, we can not exclude a potential role for this kinase in the induction of cell invasion.

Figure 12 (Next page Figure) *c-Met downstream targets involved in H. pylori-mediated cell invasion***.** AGS cells or AGS cells transfected with siRNAs directed to **(A)** c-Cbl, Gab1, Nck, and Shc, or **(B)** c-Src, FAK, PLC- γ, and Shp-2, infected or not with *H. pylori* strain 26695, at a MOI of 100, were incubated for 24 hours on Matrigel filters and assessed for invasion. Data correspond to the mean value +/- SE and are representative of three independent experiments.*, significantly different from untreated cells; **, significantly different from AGS cells non-transfected with siRNA and infected with *H. pylori* 26695. **(C)** The efficiency of the siRNA transfection was assessed by western blot. AGS cells (-), AGS tranfected with a No-silencing siRNA (NS) or with siRNA directed to c-Cbl, Gab1, Nck, Shc, c-Src, FAK, PLC- γ, and Shp-2. Staining for α-tubulin was used as loading control.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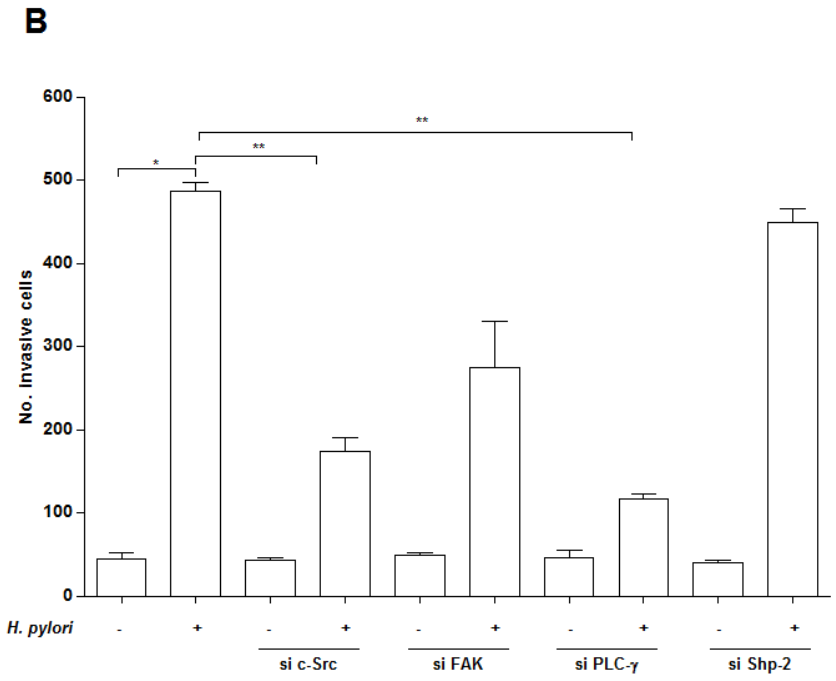
si Shc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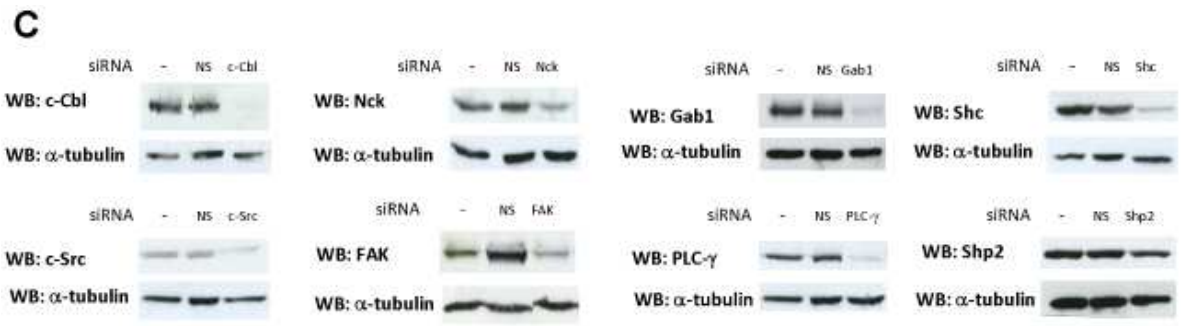
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siRNA - NS Shc
WB: Shc
WB: α-tubulin
siRNA - NS c-Src
WB: c-Src
WB: α-tubulin
siRNA - NS FAK
WB: FAK
WB: α-tubulin
siRNA - NS PLC-γ
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1.4. The role of *H. pylori* and of c-Met in Nck, c-Src and PLC-γ activation

After demonstrating that Nck, PLC-γ, and c-Src are involved in *H. pylori*-mediated cell invasion, it was next evaluated if *H. pylori* activates these molecules and whether this occurs downstream c-Met receptor activation.

To experimentally address this issue, Non-silenced and c-Met silenced AGS cells were evaluated in the presence or absence of *H. pylori*, for the activation statues of Nck, PLC-γ, and c-Src. Phosphorylation of Nck and PLC-γ was analyzed by immunoprecipitation with a general phospho-tyrosine antibody followed by western blot, and phosphorylation of c-Src was evaluated using a phospho-specific antibody for the activating Tyrosine 416. The three proteins were phosphorylated in the presence of *H. pylori* in Non-silenced cells whereas no phosphorylation could be observed in c-Met silenced cells (Figure 13). These results strongly suggest that activation of Nck, PLC-γ, and c-Src by *H. pylori* is mediated by the c-Met receptor.

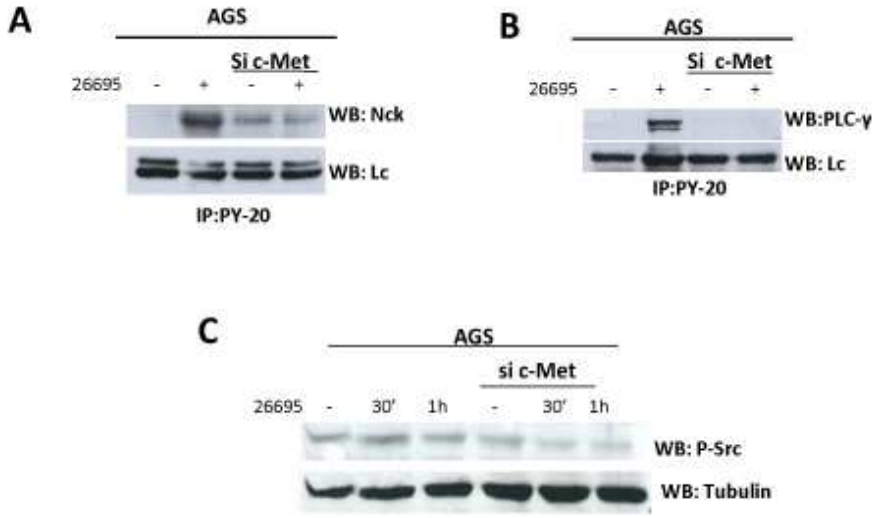


Figure 13. *H. pylori induces Nck, PLC-γ, and c-Src activation in a c-Met dependent manner***.** AGS cells or AGS cells transfected with c-Met siRNA, infected or not with *H. pylori* strain 26695, with a MOI of 100, for 1 hour were analyzed. **(A-B)** cell lysates were immunoprecitated with an antibody against tyrosine-phosphorylated residues (IP: PY-20) and immunostained with **(A)** anti-Nck or **(B)** anti-PLC-γ antibodies. **(C)** cell lysates were immunostained with an anti-phospho Tyr 416-Src antibody, and after stripping the same membrane was immunostained for α-tubulin as loading control

Overall, results presented in this Part I.1 of the Results show that *H. pylori* strains with a competent T4SS induce host gastric cell invasion through extracellular matrix

components via c-Met receptor-dependent Nck, PLC-γ, and c-Src activation.

Part I.2. Analysis of the effects of H. pylori in modulation of matrix metalloproteinase 10 (MMP-10)

Matrix metalloproteinases are a class of enzymes involved in turnover and remodeling of the extracellular matrix components and also important for cell invasion, processing growth factors and shedding of membrane bound proteins (Parks *et al.*, 2004, Chakraborti *et al.*, 2003). Since it was demonstrated in Part I.1 that *H. pylori* induces host cell invasion, and there are previous descriptions that the bacteria can regulate certain MMPs both at the level of gene expression and by proteolytic activity control (Gooz *et al.*, 2003, Bebb *et al.*, 2003, Krueger *et al.*, 2006, Oliveira *et al.*, 2006), MMP expression induced by *H. pylori* was next addressed.

2.1. Up-regulation of MMPs by *H. pylori*

The starting point of the next studies were the results of a genome-wide cDNA microarray performed by our Group (data not shown) in order to characterize changes in the gene expression profile of gastric epithelial AGS cells in response to *H. pylori* strain 60190. In this expression array, several MMPs were found to be up-regulated by *H. pylori* , namely MMP-1, MMP-7 and MMP-10 (Table 3).

Table 3: List of up-regulated MMPs after *H. pylori* infection

Matrix Metalloproteinase	Fold increase
MMP-1	6.6
MMP-7	8.6
MMP-10	14.6

A series of experiments in order to validate the results of the microarray was performed. AGS cells were infected for 24 hours with an *H. pylori* strain different from the one used in the array (*H. pylori* 26695), and the RNA isolated after infection. The mRNA levels of MMP-1, MMP-7, and MMP-10 were analyzed by qRT- PCR and compared with non-infected controls. The qRT-PCR results confirmed the array results for MMP-1, MMP-7, and MMP-10, with an up-regulation of the enzymes after *H. pylori* infection (Figure 14A,B). From the MMPs studied, MMP-10 was the one with a more pronounced increase in the

mRNA levels after infection with *H. pylori*, showing a 40-fold increase in expression in comparison with non-infected cells (Figure 14C).

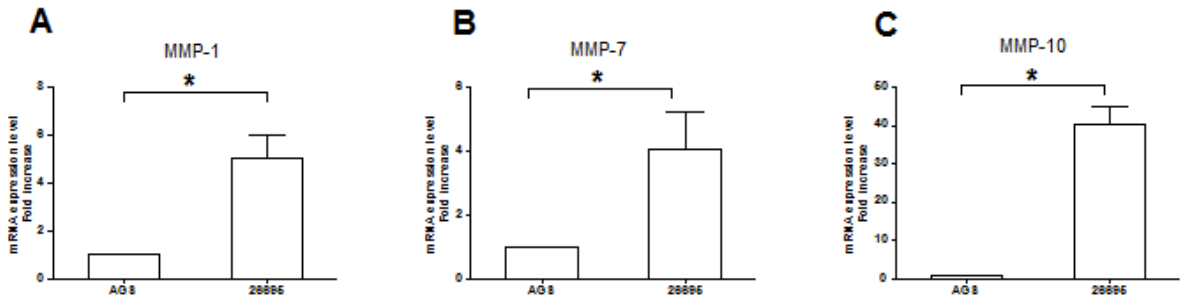


Figure 14. *H. pylori increases in MMP mRNA expression of MMP-1, MMP-7, and MMP-10*. AGS cells were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. **(A)** MMP-1, **(B)** MMP-7, and **(C)** MMP-10 expression was analyzed by qRT-PCR. MMPs expression levels were normalized to GAPDH expression and results are presented as fold difference relative to uninfected cells. Data on graphs represents the mean value +/- SE and are representative of, at least, three independent experiments. *, significantly different from non-infected cells.

2.2. Analysis of the effect of *H. pylori* in MMP-10 protein secretion and activity

Since MMP-10 showed the more pronounced increase in mRNA levels in the presence of *H. pylori*, and because the regulation of this metalloproteinase in the context of *H. pylori* infection had never been explored, attention was focused on this particular MMP.

To assess whether the increase in MMP-10 mRNA after infection is accompanied by an increase in protein secretion, the conditioned medium of AGS cells infected for 24 hours was collected and concentrated, and the levels of secreted MMP-10 were analyzed by western blot. *H. pylori* significantly enhanced the secretion of MMP-10 into the medium. (Figure 15A). Of note, no MMP-10 secretion was observed in uninfected AGS cells.

To address the enzymatic activity of secreted MMP-10, conditioned media of infected and non-infected AGS cells were analyzed using a fluorimetric assay. In this experiment, levels of active MMP-10 were calculated as a measure of cleavage of an enzyme substrate (FRET peptide with a specific sequence known to be cleaved by the active MMP-10), by changes in fluorescence emission. Compared with non-infected cells, cells

infected with *H. pylori* showed a significant increase in emitted fluorescence, reflecting an increase in MMP-10 enzymatic activity (Figure 15B).

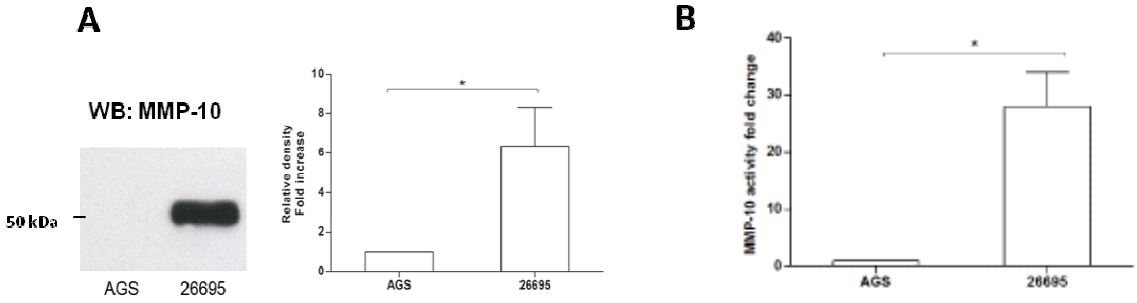


Figure 15. *H. pylori increases MMP-10 protein secretion and activity.* AGS cells were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. **(A)** Secreted MMP-10 was analyzed in conditioned media by western-blot and graphs represents the densitometry analysis of the blots. **(B)** MMP-10 activity in conditioned media was analyzed by FRET. Data on graphs represents the mean value +/- SE and are representative of three independent experiments. *, significantly different from non-infected cells.

2.3. Role of bacterial virulence in *H. pylori*-induced MMP-10 expression

To define the contribution of the CagA and of the T4SS virulence factors in *H. pylori*-mediated increase in MMP-10 mRNA, AGS cells were co-cultured for 24 hours with *H. pylori* strain 84183 and its *cag*A (84183*ΔcagA*) and T4SS (84183*ΔcagE*) mutants. The analysis of the mRNA levels of MMP-10 by qRT- PCR showed that infection with wild type strain 84183 induces an increase in the expression of MMP-10 comparable to the one induced by *H. pylori* 26695 (Figure 16). In contrast, infection with the 84183*ΔcagA* and 84183*ΔcagE* mutants, lacking *cag*A and a functional T4SS, respectively, led to the expression of significantly less amounts of MMP-10 mRNA (Figure 16). These results show that the increase in MMP-10 expression induced by *H. pylori* is dependent on the presence of a functional T4SS. Since the T4SS is needed for CagA translocation into the host cell, and because the *cag*A mutant also induced low MMP-10 expression, it is likely that *H. pylori*-mediated up-regulation of MMP-10 is CagA-dependent (Figure 16).

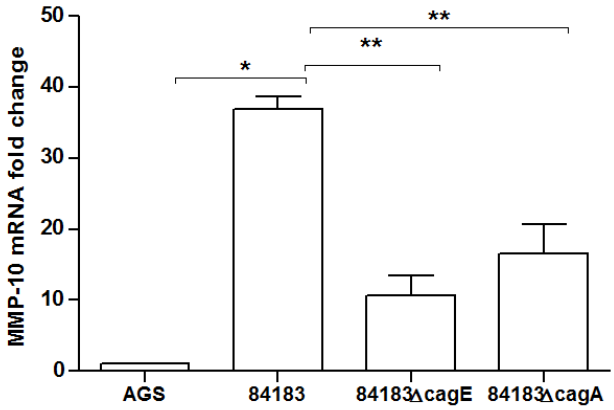


Figure 16. *H. pylori-mediated MMP-10 expression is CagA dependent*. AGS cells were infected with *H. pylori* for 24 hours at a MOI of 100 with wild-type strain 84183 and its *cag*E (84183*ΔcagE*) and *cag*A (84183*ΔcagA*) mutants. MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells. Data correspond to the mean values +/- SE and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells infected with wild-type *H. pylori* 84183.

To confirm these results, AGS cells were infected for 24 hours with a panel of clinical isolates of *H. pylori*, with known *cag*A status, as well as with the non-pathogenic and *cag*A negative strain Tx30a. In general, *H. pylori cag*A-positive strains led to a more pronounced increase in MMP-10 expression after infection than did *cag*A-negative strains (Figure 17). The main exception was strain CI-50 that, although *cag*A-positive, was not able to significantly up-regulate MMP-10 (Figure 17). It is possible that this strain has an impaired T4SS and is not able to translocate CagA into the host cells. Curiously, Tx30a was the strain that among all *cag*A-negative strains induced a more elevated level of MMP-10, which was still, much less than those induced by *cag*A-positive strains (Figure 17). These results point to an important role of CagA in MMP-10 up-regulation mediated by *H. pylor*

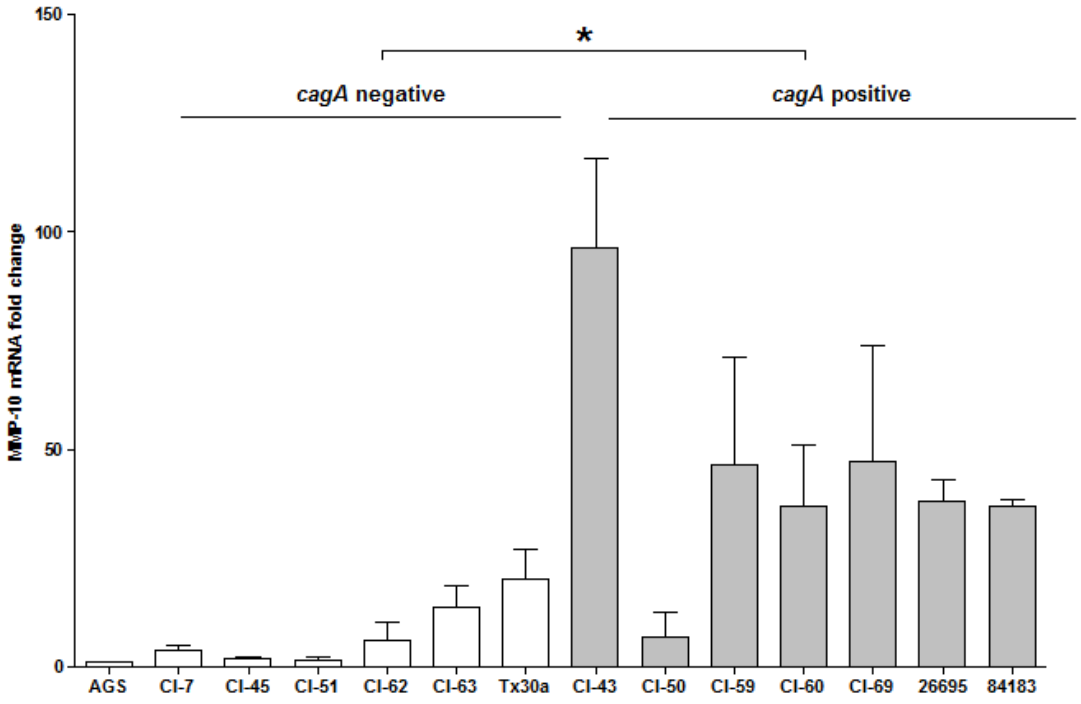


Figure 17. *MMP-10 expression is higher in cells infected with H. pylori cagA-positive than with cagA-negative strains.* AGS cells were infected for 24 hours at a MOI of 100 with a panel of *H. pylori* isolates with known *cagA* status. MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells. Data correspond to the mean values +/- SE and are representative of three independent experiments. *, significantly different from *cag*A negative strains.

2.4. MMP-10 is involved in *H. pylori*-mediated cell invasion

As one of the main functions of the MMPs is the turnover and remodeling of the extracellular matrix (Parks *et al.*, 2004), and since it was previously reported that *H. pylori cag*-positive strains induce gastric cell invasion in a context of increased MMP expression and activity (Oliveira *et al.*, 2006, Sokolova *et al.*, 2012), it was determined whether MMP-10 is involved in cell invasion mediated by *H. pylori.* AGS cells were transiently transfected with a siRNA abrogating MMP-10 expression, and the efficiency of the knockdown was accessed by qRT-PCR. Invasion assays were performed 24 hours after transfection, by incubating AGS cells with *H. pylori* on Matrigel filters for an additional 24 hours. Silencing of MMP-10 inhibited *H. pylori*-induced MMP-10 expression in AGS cells (Figure 18A), and signifincantly inhibited cell invasion in response to *H. pylori* infection

(Figure 18B). These findings demonstrate that MMP-10 plays a role in *H. pylori*-mediated cell invasion.

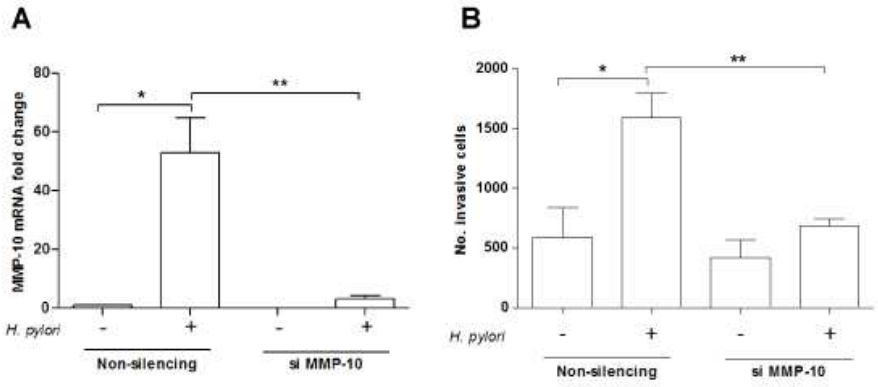


Figure 18. ***MMP-10 is involved in H. pylori-mediated cell invasion.*** **(A)** AGS cells were treated with a Non-silencing siRNA or a siRNA directed to MMP-10, and co-cultured with *H. pylori* for 48 hours. The efficiency of the knockdown was evaluated by qRT-PCR. **(B)** AGS cells treated with a Non-silencing or a siRNA directed to MMP-10, were co-cultured with *H. pylori* for 24 hours at a MOI of 100 on Matrigel coated filters. Data on graph represents the mean value +/- SE and are representative of three independent experiments. *, significantly different from non-infected cells;**, significantly different from cells infected with *H. pylori* 60190.

2.5. Role of c-Met and EGFR in *H. pylori*-induced MMP-10 expression

It has been reported that MMPs may be up-regulated by growth factors that activate RTKs (McCawley *et al.*, 2001, Egeblad *et al.*, 2002). c-Met and EGFR are two RTKs known to be activated during *H. pylori* infection (Wallasch *et al.*, 2002, Churin *et al.*, 2003, Oliveira *et al.*, 2006). Additionally, EGFR was shown to be implicated in MMP-10 up-regulation in squamous cell carcinoma of head and neck (Wilkins-Port *et al.*, 2007). Therefore, the next experiments were performed to address whether c-Met and EGFR are implicated in *H. pylori*-mediated MMP-10 expression.

First, it was tested whether stimulation of AGS cells with HGF and EGF, the natural ligands of c-Met and EGFR respectively, induced differences in MMP-10 expression. AGS cells were treated with HGF at 250 ng/mL and EGF at 50 ng/mL for 24 hours. The RNA was isolated from those cells and MMP-10 expression was analyzed by qRT- PCR. Both HGF and EGF enhanced MMP-10 expression, with EGF inducing a more pronounced increase (Figure 19A,B). These experiments showed that MMP-10 can be up-regulated in AGS cells via c-Met and EGFR. Next the role of these two RTKs in the

induction of MMP-10 during infection was addressed. To achieve this aim, AGS cells were treated with a Non-silencing siRNA or with siRNA directed to c-Met, and with a chemical inhibitor of EGFR, AG-1478, or with DMSO alone, and infected with *H. pylori* or left untreated for 24 hours. The downregulation of c-Met and EGFR abrogated *H. pylori*-mediated MMP-10 expression (Figure 19C,D). These findings indicate that the increase in MMP-10 expression induced by *H. pylori* is mediated by the activation of c-Met and EGFR.

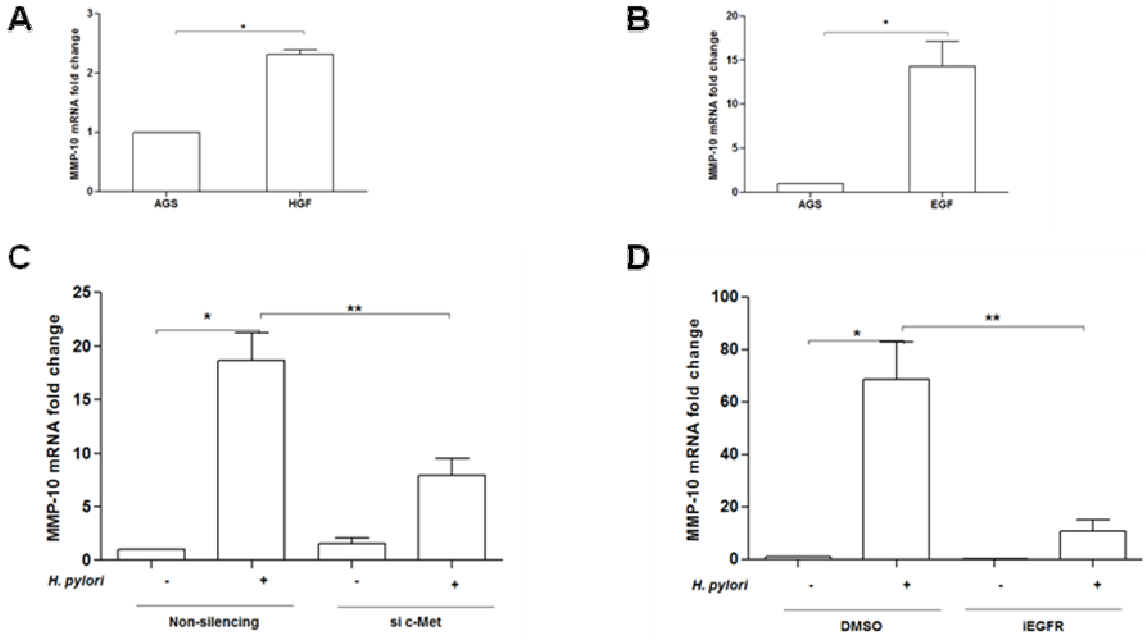
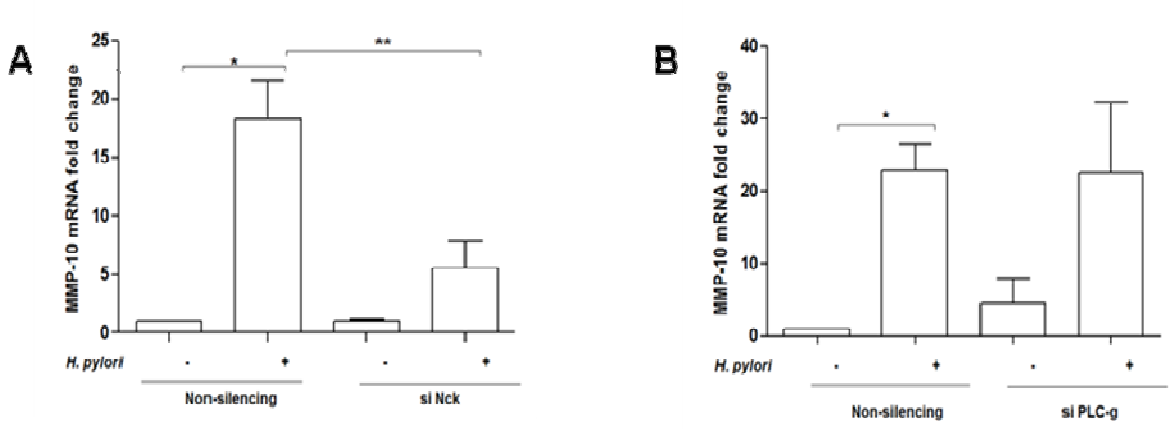


Figure 19. *H. pylori increases MMP-10 expression via EGFR and c-Met.* AGS cells were treated with HGF at 250 ng/mL **(A)** and EGF at 50 ng/mL **(B)** for 24 hours. **(C-D)** AGS cells were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100, after the transient tranfection with a Non-silencing siRNA (NS) or an siRNA directed to c-Met (si c-Met) **(C)**, or after treatment with the EGFR chemical inhibitor AG-1478 at a final concentration of 5µM (iEGFR), or with DMSO, 1 hour before the infection **(D)**. **(A-D)** MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells **(A-B)**, cells treated with a Non-silencing siRNA **(C)** or with DMSO **(D)**. Data correspond to the mean values +/- SE and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from non-silenced cells or cells treated with DMSO infected with wild type *H. pylori* strain.

2.6. Role of c-Met and EGFR downstream targets in *H. pylori*-induced MMP-10 expression

In order to have a more clear picture of the molecules involved in c-Met and EGFR signaling that lead to MMP-10 up-regulation in *H. pylori* infection, and because in Part I.1 several c-Met downstream targets that also function downstream EGFR were found to be important for *H. pylori*-induced cell invasion, the involvement of Nck, PLC-γ, and c-Src in *H. pylori*-induced MMP-10 expression was addressed.

To fulfill this aim, the expression of Nck, PLC-γ, and c-Src in AGS cells was downregulated using siRNAs, and cells were infected for 24 hours with *H. pylori*. As a control, AGS cells were transfected with a Non-silencing siRNA. Since the silencing of c-Src was difficult to achieve, the chemical inhibitor PP2 directed to the Src family kinases was also used. Cells were treated one hour before infection with PP2 or DMSO and then infected for 24 hours. RNA was isolated after this period and analyzed by qRT-PCR. As expected, MMP-10 expression increased in AGS cells infected with *H. pylori*, but this increase was attenuated in cells silenced for Nck (Figure 20A). Similarly *H. pylori*-induced MMP-10 expression was decreased in cells silenced for c-Src or in which c-Src activity was inhibited with PP2 (Figure 20C,D). In contrast, the silencing of PLC-γ had no effect in *H. pylori*-mediated MMP-10 expression (Figure 20B). These results point to a role for Nck and c-Src in the regulation of MMP-10 during infection.



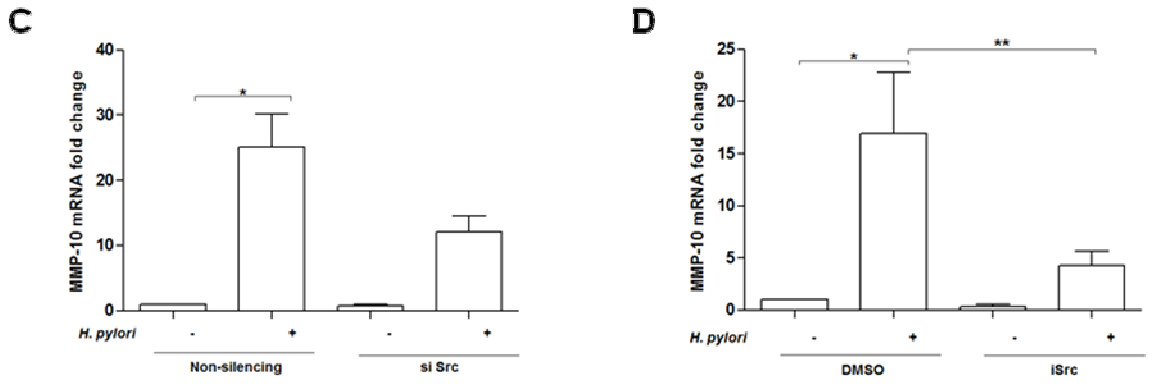


Figure 20. *Nck and c-Src, but not PLC-γ, are involved in H. pylori-mediated MMP-10 expression* **(A-C)** AGS cells alone or transiently transfected with a Non-silencing siRNA (NS) or with siRNAs directed to Nck, PLC-γ and c-Src, or **(D)** after a treatment for 1hour with the Src family kinases chemical inhibitor PP2 (iSrc), or DMSO, were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to cells treated with a Non-silencing siRNA **(A-C)** or with DMSO **(D)**. Data correspond to the mean values +/- SE and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from non-silenced cells or cells trated with DMSO infected with a wild type *H. pylori* strain.

2.7. Analysis of the participation of ERK1/2, JNK and p38 signaling pathways in *H. pylori*-mediated MMP-10 expression

Because ERK, JNK, and p38 pathways are involved both in the induction of different MMPs (Ridley *et al.*, 1997, Reunanen *et al.*, 1998) and in *H. pylori*-driven signaling (Naumann *et al.*, 1999, Wessler *et al.*, 2000), and as they can function downstream c-Met and EGFR (Terakado *et al.*, 2011, Brusevold *et al.*, 2012, Tsai *et al.*, 2012), it was next determined if these pathways were involved in *H. pylori*-mediated MMP-10 expression. For this purpose, the effect of chemical inhibitors of ERK1/2 (U-0126), JNK (SP600125), and p38 (SB203580) on MMP-10 expression after infection was evaluated. AGS cells were treated with the inhibitors or with the vehicle (DMSO) alone, 1 hour before infection. Cells were then infected with *H. pylori* 26695 for 24 hours in the presence of the inhibitors, and MMP-10 expression was measured by qRT-PCR. Infection with *H. pylori* in the presence of DMSO did not affect MMP-10 expression stimulated by the bacteria alone (Figure 21). The treatment with the ERK1/2 inhibitor resulted in complete abrogation of MMP-10 expression and treatment with the JNK inhibitor led to a significant decrease of MMP-10 expression after *H. pylori* infection (Figure 21A). On the contrary, the inhibition of

p38 resulted in enhanced expression of MMP-10 after infection (Figure 21B). Taken together, these results suggest that MMP-10 expression induced by *H. pylori* is partially mediated by JNK and ERK1/2 pathways, and that p38 may have an inhibitory role in this process.

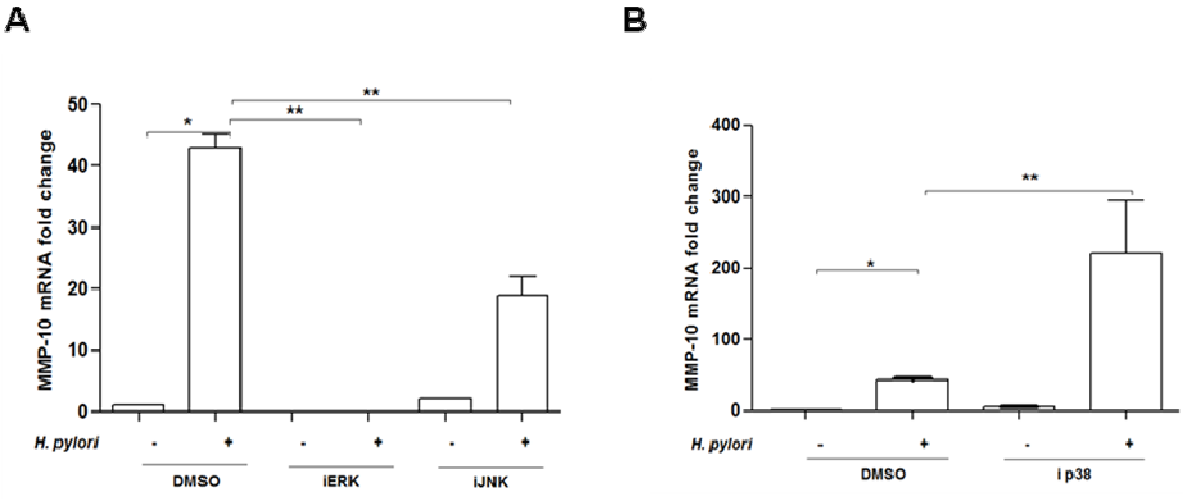


Figure 21. *H. pylori increases MMP-10 expression through ERK and JNK pathways.* AGS cells were treated 1 hour before the infection with DMSO or with chemical inhibitors of ERK1/2 (U-0126, 25 µM; iERK) **(A)**, JNK (SP600125, 20 µM; iJNK) **(A)**, and p38 (SB203580, 20 µM; ip38) **(B)**. **(A-B)** After treatment AGS cells were infected with *H. pylori* 26695 for 24 hours at a MOI of 100 (Hp). MMP-10 expression was evaluated by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells treated with DMSO. Data correspond to the mean values ± SE and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells treated with DMSO infected with a wild type *H. pylori* strain.

2.8. Analysis of MMP-10 modulation by cytokines produced by *H. pylori* infection

Several cytokines are able to modulate MMP secretion in pathological contexts such as chronic inflammatory diseases (Zenmyo *et al.*, 1996). Since the infection with *H. pylori* induces chronic gastritis and it is known that pro-inflammatory cytokines are produced in response to *H. pylori* infection, it was next evaluated whether these cytokines play a role in the modulation of MMP-10 expression.

A panel of pro- and anti-inflammatory cytokines available in the laboratory was used. AGS cells were treated for 24 hours with recombinant IL-1β, TNF-α, TGF-β, and IL-10, at a final concentration of 10 ng/mL, with IL-8 at 8 ng/mL, and with INF-γ at 100U/mL.

After this period, RNA was isolated and MMP-10 expression was analyzed by qRT-PCR. The graph on Figure 22 shows that from the panel of cytokines tested, only IL-1β was able to induce a significant increase in MMP-10 expression. This suggests that not only *H. pylori*, but also IL-1β that is produced in the gastric mucosa in response to the infection may contribute to MMP-10 modulation.

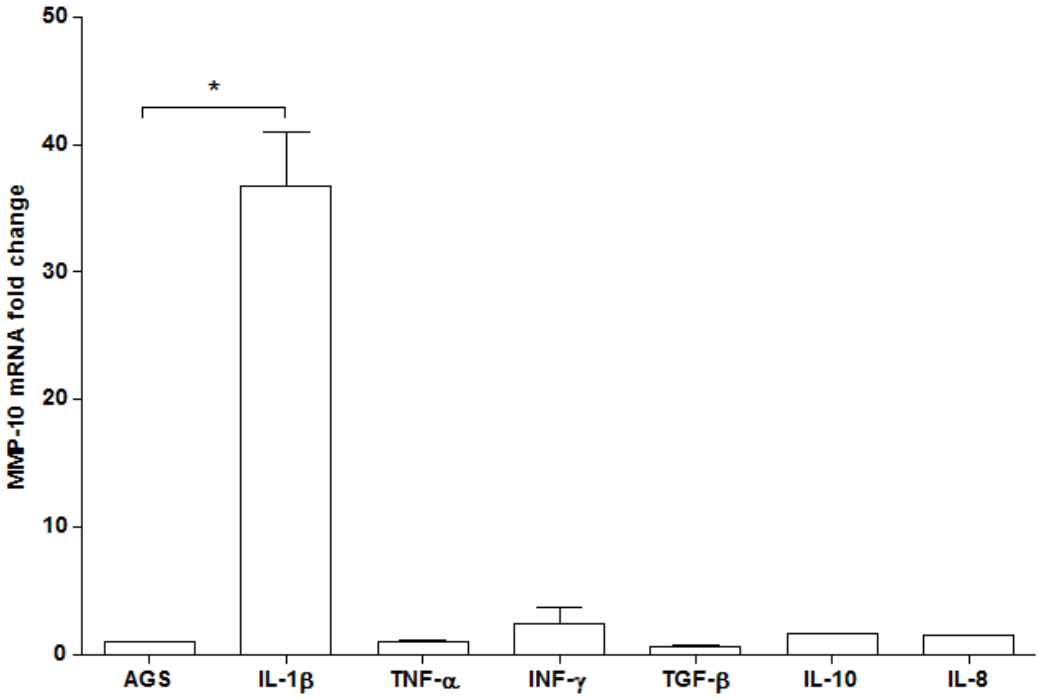


Figure 22. *IL-1β treatment increase MMP-10 expression.* AGS cells were treated with IL-1β, TNF-α, TGF-β and IL-10 at a final concentration of 10 ng/mL and, with IL-8 at 8 ng/mL and INF-γ at 100U/mL for 24 hours. MMP-10 expression was evaluated by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to untrated cells. Data on graphs represents the mean value +/- SE and are representative of three independent experiments, except for IL-10 and IL-8 with two independent experiments. *, significantly different from non-treated cells.

2.9. Role of MMP-10 in MMP-1 activation in *H. pylori* infection

Several MMPs are activated via proteolytic cleavage by other MMPs (Mannello *et al.*). MMP-10 can activate other MMPs, namely MMP-1 (Nakamura *et al.*, 1998, Windsor *et al.*, 1993), a metalloproteinase that is up-regulated by *H. pylori,* and is involved in gastric tissue degradation and remodeling (Krueger *et al.*, 2006, Pillinger *et al.*, 2007, Sokolova *et al.*, 2012). Indeed, in our model system, MMP-1 was also found to be up-regulated in AGS cells by *H. pylori* infection (Figure 23).

Therefore, and to study whether MMP-10 has a role in MMP-1 activation during *H. pylori* infection, AGS cells were transiently transfected with a siRNA directed to MMP-10 or with a Non-silencing siRNA, and infected for 24 hours with *H. pylori* strain 26695. After the infection period, the conditioned media were collected and concentrated, and the levels of secreted MMP-1 were analyzed by western-blot. As expected, *H. pylori* infection led to an increase in the levels of secreted MMP-1 (Figure 23). Interestingly, the silencing of MMP-10 resulted in a decrease in the secreted form of MMP-1 (Figure 23). These results suggest that MMP-10 may function as a pro-MMP-1 activator in the context of *H. pylori* infection.

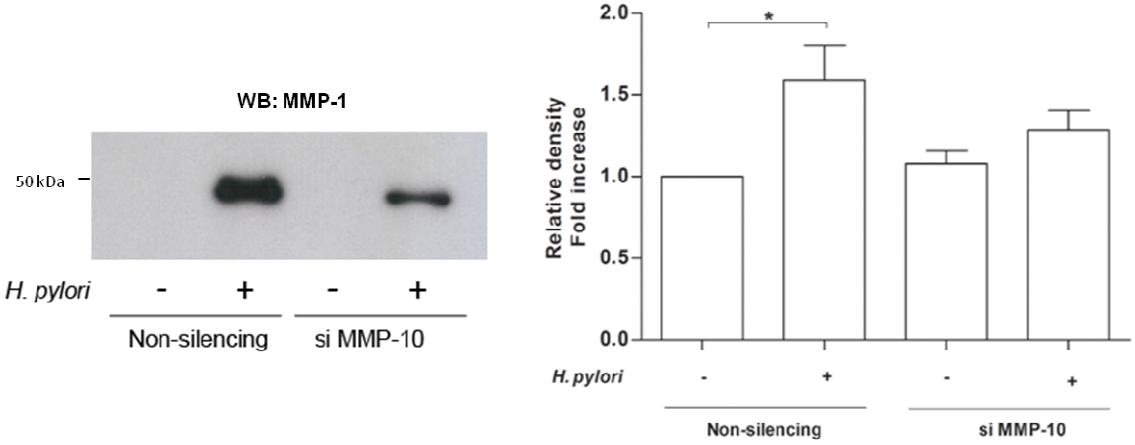


Figure 23. *MMP-10 mediates MMP-1 activation during H. pylori infection.* AGS cells transiently transfected with Non-silencing siRNA (NS) or with siRNA directed to MMP-10 (si MMP-10) were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. **(A)** Secreted MMP-1 in conditioned media was analyzed by western-blot. **(B)** Data on graphs, corresponding to the quantification of the blots, represents the mean value ± SE, and are representative of three independent experiments. *, significantly different from uninfected cells.

Part II

Part II.1. Analysis of the effects of E-cadherin in H. pylori-mediated cell invasion and signaling

The E-cadherin-catenin complex is a major component of the adherens junctions and functions has an invasion suppressor. The function of E-cadherin is frequently compromised in gastric carcinoma, a condition strongly associated with *H. pylori* infection. Results from the Part I of the Results together with published data, showed that *H. pylori* activates the c-Met receptor leading to a motogenic response and to invasion of host cells (Churin *et al.*, 2003). Therefore, in Part II of the Results it was investigated whether E-cadherin has a role in *H. pylori*-induced c-Met phosphorylation-dependent cell invasion.

1.1. *H. pylori*-mediated invasive phenotype in cells with intact adherens junctions

The human gastric carcinoma cells AGS, IPA220, and NCI-N87 were confronted with *H. pylori* on Matrigel invasion assays. It was observed that, in contrast to AGS cells, which acquired an invasive phenotype in response to *H. pylori*, IPA220 and NCI-N87 cells were significantly less responsive to *H. pylori* (Figure 24). From the panel of gastric cell lines tested, IPA220 and NCI-N87 cells have, to the best of our knowledge, intact E-cadherin/catenin complexes, whereas AGS cells harbour an E-cadherin mutation leading to a truncated form of the protein that is not expressed. These results point to a role of the E-cadherin/catenin complex in the inhibition of *H. pylori*-mediated invasion.

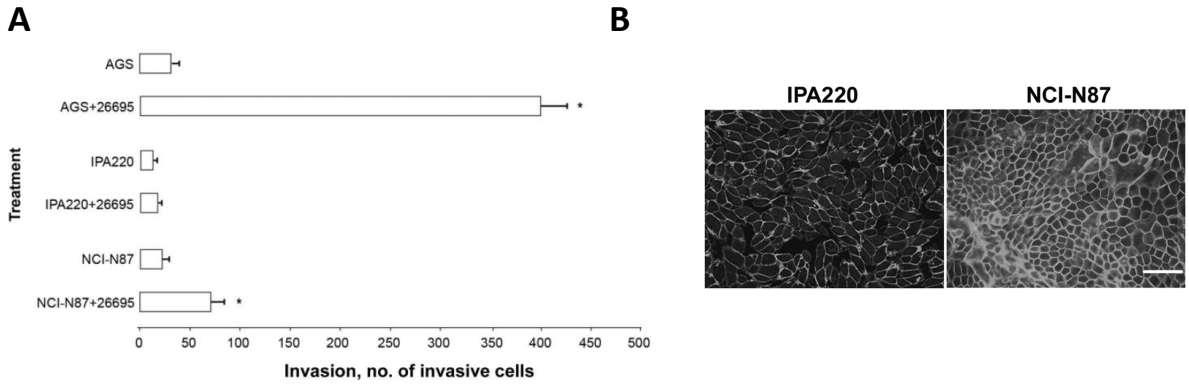


Figure 24. *The E-cadherin/catenin complex suppresses H. pylori-mediated cell invasive phenotype.* **(A)** Invasion assays of AGS, IPA220 and NCI-N87 cells infected or not for 24 hours with *H. pylori* on Matrigel-coated filters. Graphics represent the mean value of invasion ± SD and are representative of three

independent experiments. *, significantly different from uninfected cells. **(B)** E-cadherin staining of IPA220 and NCI-N87 cells. Nuclei were counterstained with DAPI. Scale bar represents 100 μm.

1.2. Generation and characterization of an AGSEcad cell line

To investigate the role of E-cadherin in *H. pylori*-mediated invasive phenotype, AGS cells that do not express this adhesion molecule were transduced with wild-type E-cadherin. In this way it was possible to compare the role of *H. pylori* infection in two cell lines with the same genetic background and that only differ in E-cadherin expression. AGS were stably transduced with the human *CDH1* gene using a lentivirus transduction system. For clone selection, transduced cells were grown in medium with blasticidin. Only clones with a homogeneous expression of E-cadherin at the cell membrane were selected. To exclude clonal dependency, all experiments reported were performed with two distinct clones (clones 1a and 4b).

E-cadherin expression was confirmed by western blot and immunofluorescence (Figure 25). Immunocytochemistry of the E-cadherin-transduced AGS cells (AGSEcad) showed E-cadherin, β-, α-, and p120-catenins mostly located at the cell membrane in a honey-comb-like pattern (Figure 25B), suggesting that E-cadherin recruits β-, α-, and p120-catenin to this site to form an adhesion complex.

To elucidate the functionality of the E-cadherin/catenin complex in the AGSEcad cells, it was performed an aggregation assay, a standard method that evaluates the ability of cells to aggregate in an E-cadherin-dependent manner. AGS cells that do not express E-cadherin are not able to aggregate, remaining as single cells after 48 hours of incubation (Figure 26). In contrast, AGSEcad cells form small aggregates, suggesting the presence of a functional E-cadherin/catenin complex. The fact that these cells are no longer able to aggregate in the presence of the MB2 antibody that inhibits E-cadherin-homophilic interactions and consequently cell-cell adhesion, confirms that the E-cadherin/catenin complex of AGSEcad cells is functional.

These set of experiments demonstrate that in AGS transduced with E-cadherin, β-, α-, and p120-catenins are recruited to the membrane, where they assemble a functional adhesion complex.

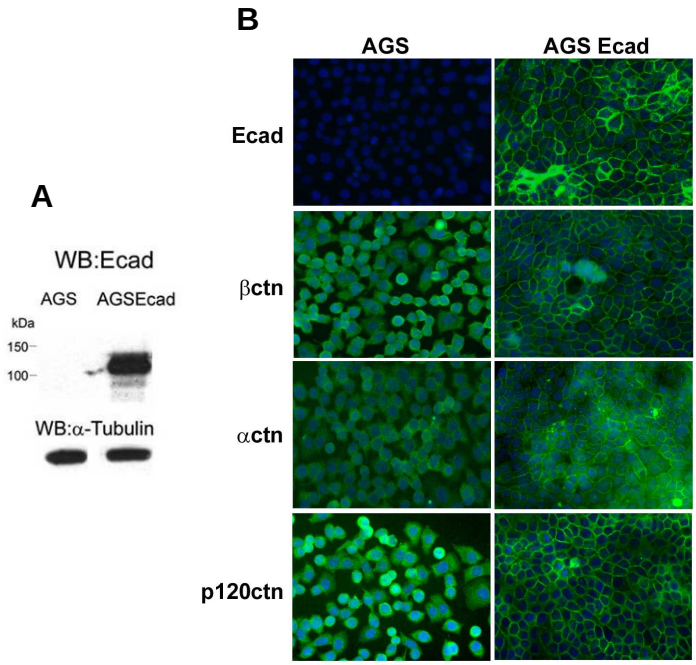


Figure 25 - *Characterization of AGS cells stably expressing E-cadherin.* **(A)** E-cadherin expression was evaluated in AGS and AGSEcad cell lines by western blot for E-cadherin and α-tubulin immunostaining was used as loading control. **(B)** Immunoflurescence for the adherens junctions components E-cadherin, β-, α- or p120-catenin of AGS and AGSEcad cells. Nuclei were counterstained with DAPI.

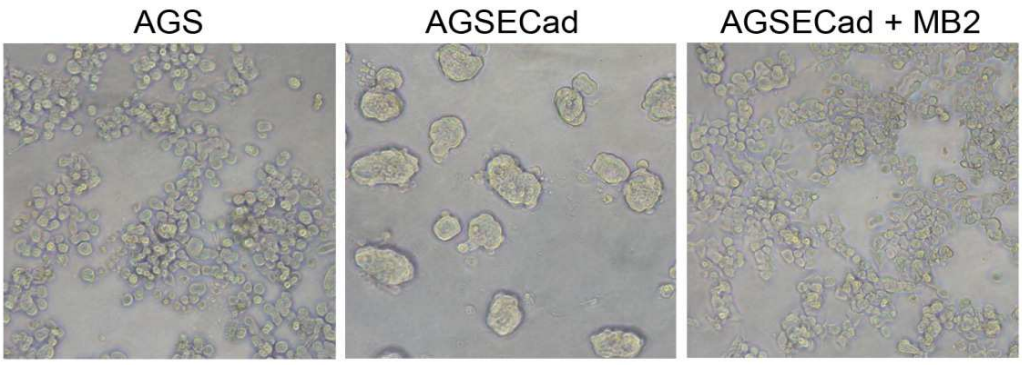


Figure 26 – *Slow aggregation assay in AGS and AGSEcad cell lines*. Aggregation assay of AGS, AGSEcad, and AGSEcad cells cultured with an anti-E-cadherin antibody (AGSEcad+MB2). Cells were plated isolated on top of agar and evaluated after 48h. Figures are representative of three independent experiments.

1.3. Role of E-cadherin in *H. pylori*-mediated cell invasion

Giving that AGSEcad cells have a functional adherens junctions complex, it was next investigated whether E-cadherin was sufficient to inhibit *H. pylori*-mediated invasive phenotype.

AGS, AGSEcad, and NCI-N87 cells were infected with *H. pylori* strains 26695 and 60190, and used in Matrigel invasion assays. After infection, AGSEcad cells displayed significantly lower levels of invasion than AGS cells (Figure 27). Similar results were obtained in infected NCI-N87 cells that endogenously express E-cadherin, suggesting that E-cadherin expression is sufficient to suppress *H. pylori*-mediated invasive phenotype.

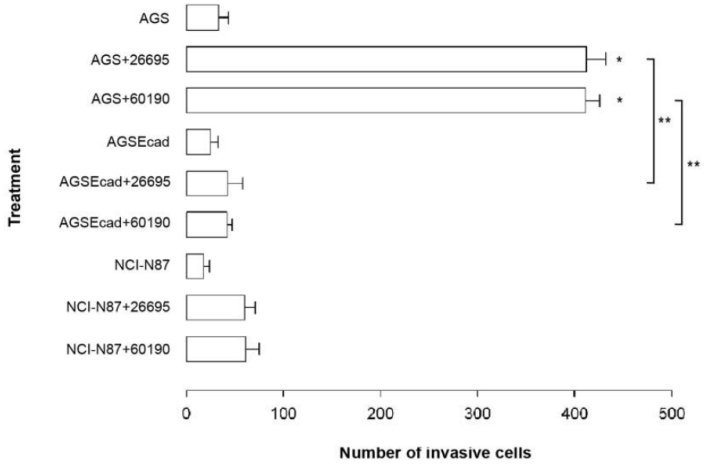


Figure 27. *E-cadherin counteracts H. pylori-mediated cell invasive phenotype.* AGS, AGSEcad, and NCI-N87 cells infected or not for 24 hours with *H. pylori* strain 26695 or 60190 on Matrigel-coated filters. Graphics represent the mean value of invasion +/- SD and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from AGS cells infected with *H. pylori*.

1.4. Role of E-cadherin in *H. pylori*-mediated c-Met and p120-catenin tyrosine phosphorylation

Since the cell motogenic response and invasion induced by *H. pylori* in AGS cells require c-Met phosphorylation (Churin *et al.*, 2003, Oliveira *et al.*, 2006), the effect of E-cadherin on the phosphorylation status of c-Met was investigated. In addition and because p120-catenin tyrosine phosphorylation is modulated by E-cadherin (Ozawa *et al.*, 2001), the phosphorylation status of p120-catenin was also investigated.

Lysates of *H. pylori*-infected AGS and AGSEcad cells were immunoprecipitated with an antibody recognizing tyrosine-phosphorylated residues, and immunoblotted with anti-c-Met and anti-p120-catenin antibodies (Figure 28).

The expression of c-Met and of p120-catenin was not altered by *H. pylori* in any of the cell lines. In AGS cells, *H. pylori* increased the phosphorylation level of c-Met and of p120-catenin. In contrast, in AGSEcad cells *H. pylori* decreased the phosphorylation levels of both proteins. These results were further confirmed in the NCI-N87 cell line, where tyrosine phosphorylation levels of c-Met and p120-catenin decreased after infection (Figure 28). Overall, these results suggest that E-cadherin suppresses *H. pylori*-mediated phosphorylation of c-Met and of p120-catenin.

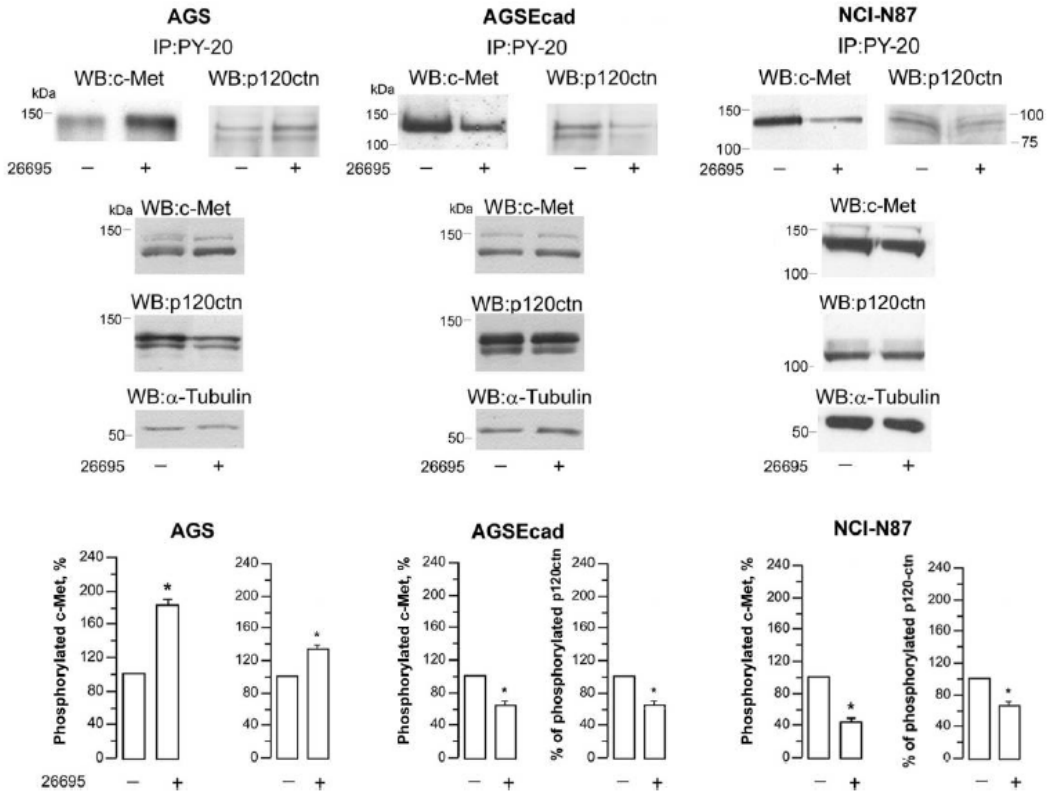


Figure 28. *E-cadherin counteracts H. pylori-mediated c-Met and p120-catenin tyrosine phosphorylation.* Western blot analysis of AGS, AGSEcad and NCI-N87 cells infected with *H. pylori* for 1 hour. Total cell lysates were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (PY-20) and immunostained with anti-c-Met or anti-p120-catenin antibodies. In parallel, total cell lysates were immunostained with the same antibodies to control differences of expression. α-tubulin immunostaining was used as loading control. Graphics represent the variation in c-Met and p120-catenin tyrosine phosphorylation in comparison to the endogenous phosphorylation levels of uninfected cells. Data correspond to the mean value +/- SD and are representative of three independent experiments. *, significantly different from uninfected cells.

1.5. Effect of *H. pylori* on the localization of elements of E-cadherin-catenin complex

Using confocal microscopy, the localization of the elements of the E-cadherin/catenin complex after *H. pylori* infection was analysed. One hour after infection with *H. pylori*, there was an increase in the intensity of membrane staining of E-cadherin and of p120-catenin (Figure 29), and in the nuclear levels of β-catenin (data not shown). No differences regarding α-catenin were observed.

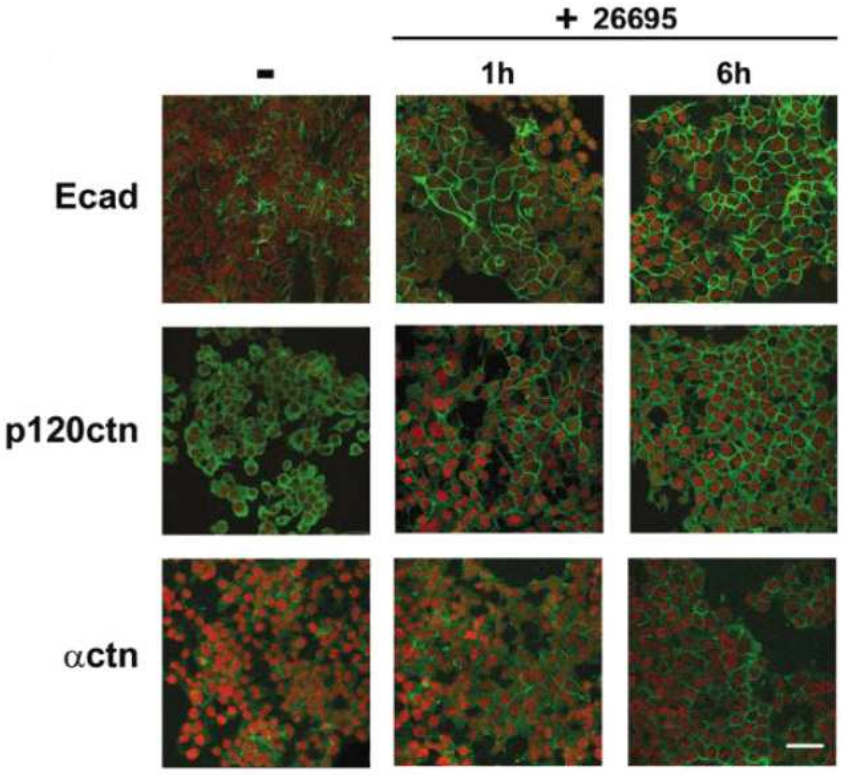


Figure 29. *H. pylori affects the localization of elements of the E-cadherin/catenin complex.* AGSEcad monolayers were infected with *H. pylori* for 1 or 6 hours. After fixation, monolayers were stained with antibodies specific for E-cadherin, p120- or α-catenins (green). Nuclei were counterstained with DAPI (red). Scale bar represents 40 μm. Images are representative of three independent experiments.

Since CagA localizes to the inner surface of the plasma membrane after injection (Segal *et al.*, 1999, Asahi *et al.*, 2003), and the membrane staining of E-cadherin and p120-catenin increased after infection (Figure 29), the putative co-localization between CagA and these E-cadherin/catenin complex elements was investigated.

Analysis of the xz and yz confocal sections suggest that after 1 hour of infection of AGSEcad cells, CagA partially co-localizes with E-cadherin and with p120-catenin

(arrowheads). Immunofluorescent signal corresponding to CagA was also visualized in areas negative for p120-catenin and E-cadherin (Figure 30). Specificity of the immunostaining was confirmed with non-immunizing IgGs of the same isotype.

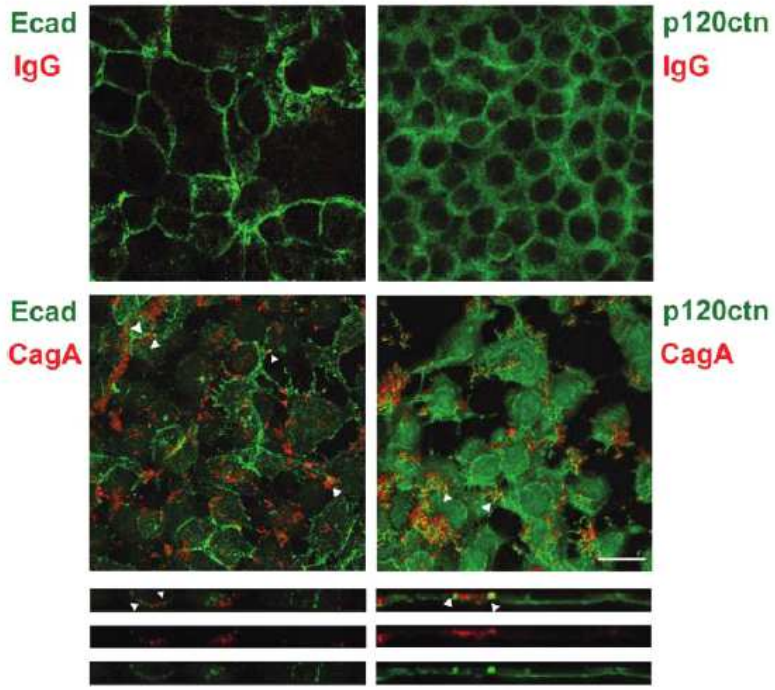


Figure 30. *CagA, E-cadherin and p120-catenin co-localization during infection.* AGSEcad monolayers infected with *H. pylori* for 1 hour were double immunostained for E-cadherin (green) and CagA (red) or p120-catenin (green) and CagA (red). Immunostainings were also performed using a CagA isotype antibody as control. xz or yz sections for both channels or for each channel apart can be visualized in more detail below each panel. Arrowheads indicate areas of co-localization. Images were obtained with a confocal microscope and are representative of two independent experiments. Scale bar represents 10 µm.

1.6. Analysis of *H. pylori* CagA interactions with E-cadherin and with p120-catenin

Since *H. pylori* CagA interacts and co-localizes with elements of the E-cadherin/catenin complex, the role of *H. pylori* virulence protein CagA in this interaction was investigated. Lysates of *H. pylori*-infected AGSEcad cells were immunoprecipitated with anti-E-cadherin or anti-p120 catenin antibodies, and immunoblotted with an anti-CagA antibody. It was observed that CagA co-immunoprecipitated with E-cadherin and, interestingly, it was also observed that CagA co-immunoprecipitated with p120-catenin (Figure 31A).

Immunoprecipitation with non-immunizing IgGs of the same isotype confirmed the specificity of such interactions. Likewise, in NCI-N87 cells CagA imunoprecipitated with E-cadherin and also with p120-catenin (Figure 31A). Furthermore, in both AGSEcad and NCI-N87 cells, in the presence of bacteria the binding between E-cadherin and p120-catenin was enhanced (Figure 31B,C). No differences were observed regarding the β-catenin/α-catenin interaction. Thus, our results indicate that after infection, *H. pylori* CagA binds to elements of the E-cadherin/catenin complex.

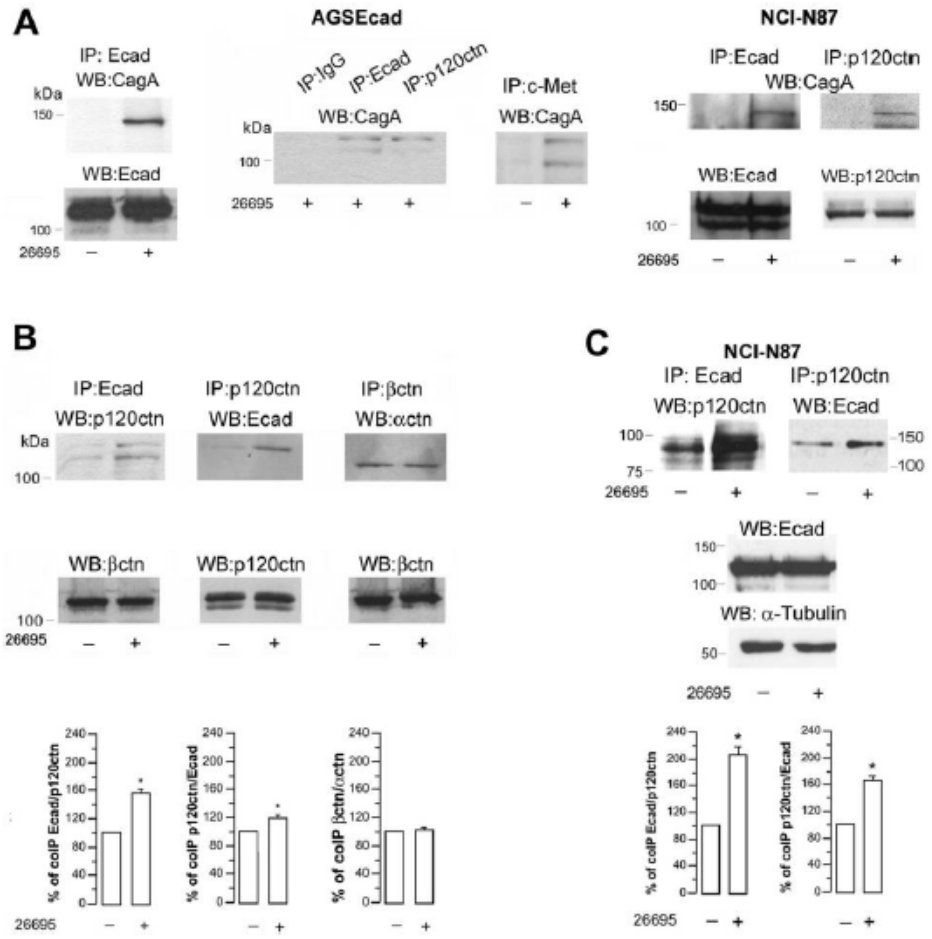


Figure 31. *H. pylori CagA interacts with E-cadherin, with p120-catenin, and with c-Met*. **(A)** AGSEcad and NCI-N87 cells were infected with *H. pylori* for 1 hour. Cell lysates were immunoprecipitated with anti-E-cadherin, anti-p120-catenin or anti-c-Met antibodies, and used for Western blot to be immunostained with an anti-CagA antibody. Immunoprecipitation was also performed with the same isotype control IgGs. Immunoblots were restained for E-cadherin or p120-catenin as loading controls. **(B)** AGSEcad cells were infected with *H.* pylori for 1 hour. Cell lysates were immunoprecipitated with anti-E-cadherin, anti-p120-catenin or anti-β-catenin antibodies, and used for Western blot to be immunostained with anti-p120-catenin, anti-E-cadherin, and anti-α-catenin antibodies. Immunoblots were restained for p120- or β-catenins, as loading

controls. **(C)** NCI-N87 cells were infected with *H. pylori* for 1 hour. Cell lysates were immunoprecipitated with anti-E-cadherin or anti-p120-catenin antibodies, and used for Western blot to be immunostained with the same antibodies. Immunoblot was restained for E-cadherin as loading control. Graphics represent the variation in protein co-immunoprecipitation in comparison to the endogenous levels of uninfected cells. Data correspond to the mean value +/- SD and are representative of three independent experiments. *, significantly different from uninfected cells.

Since CagA targets the c-Met receptor, inducing its phosphorylation (Churin *et al.*, 2003, Oliveira *et al.*, 2006), and activating downstream effector molecules, it was next examined whether the interactions between CagA and E-cadherin, and CagA and p120-catenin occurred downstream c-Met.

Co-immunoprecipitation studies on *H. pylori*-infected AGSEcad cells confirmed that CagA interacts with c-Met (Figure 31A) as previously described (Churin *et al.*, 2003). Interestingly, interactions established between c-Met and E-cadherin, and between c-Met and p120-catenin were enhanced after infection with *H. pylori* 26695 and 60190 (Figure 32A). In agreement with these observations, in *H. pylori*-infected NCI-N87 cells, increased binding of c-Met and E-cadherin and of c-Met and p120-catenin was also observed (Figure 32A). When cells were infected with a *cagA* mutant strain (60190CagA$^-$), there was a decrease in c-Met/E-cadherin but not in c-Met/p120-catenin interactions (Figure 32B), suggesting that CagA plays a role in the formation of the c-Met/E-cadherin interaction.

Studies with siRNA targeting c-Met did not affect the expression of p120-catenin and, as expected, abolished the interactions between c-Met and p120-catenin (Figure 32C) and between c-Met and E-cadherin (data not shown). Noteworthy, silencing of c-Met abolished the interactions between CagA and p120-catenin and also between CagA and E-cadherin (Figure 32D). These results suggest that interactions established between CagA and E-cadherin and between CagA and p120-catenin are c-Met dependent.

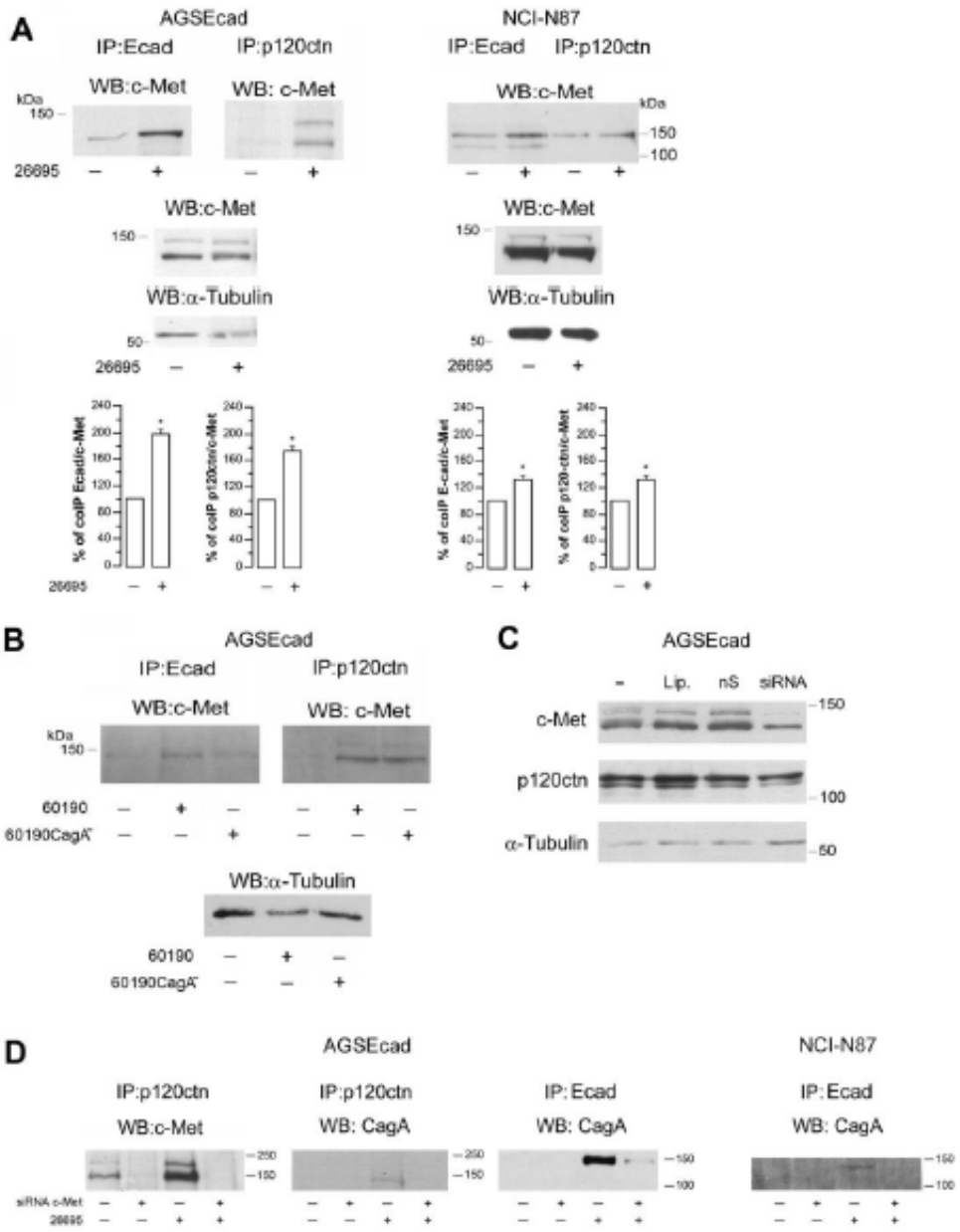


Figure 32. *The interaction established between CagA, E-cadherin, and p120-catenin is mediated by c-Met.* **(A)** AGSEcad and NCI-N87 cells were infected with *H. pylori* for 1 hour and cell lysates were immunoprecipitated with anti-E-cadherin or anti-p120-catenin antibodies and used for Western blot to be immunostained with an anti-c-Met antibody. Anti-c-Met and anti- α-tubulin antibodies were used as loading controls. **(B)** AGSEcad cells were infected with *H. pylori* strain 60190 and with its *cagA* mutant (60190CagA⁻) for 1 hour. Cell lysates were immunoprecipitated with anti-E-cadherin or anti-p120-catenin antibodies and used for Western blot to be immunostained with an anti-c-Met antibody. Anti-c-Met and anti-α-tubulin antibodies were used as loading controls. **(C)** AGSEcad cells were transiently transfected with siRNA directed to c-Met. The effect of transfection on c-Met, p120-catenin and α-tubulin expression was evaluated by Western blot with specific antibodies. **(D)** AGSEcad and NCI-N87 cells transfected or not with c-Met siRNA were infected with *H. pylori* for 1 hour. Cell lysates were immunoprecipitated with anti-p120-catenin and anti-E-cadherin antibodies and

used on Western blot to be immunostained with anti-c-Met, and anti-CagA antibodies. Data correspond to the mean value +/- SD and are representative of three independent experiments. *, significantly different from uninfected cells.

Altogether, the results presented in Part II of the Results evidence that *H. pylori* alters the localization of elements of the E-cadherin/catenin complex, leading to formation of a multiproteic complex composed by CagA, c-Met, E-cadherin, and p120-catenin. The formation of this complex impairs c-Met and p120-catenin tyrosine phosphorylation and suppresses the cell invasive phenotype induced by *H. pylori*.

DISCUSSION

H. pylori induces alterations in several host cell signaling transduction pathways, which results in alterations of the normal cell behavior. The host cell invasive phenotype induced by *H. pylori* is a not well studied issue, and the clarification of its molecular mechanisms as well as the consequences to the cell and tissue homeostasis is of great importance. The insights that a study in this area can bring may be of great importance to understand the pathological conditions associated with *H. pylori* infection.

Part I. Analysis of the effects of *H. pylori* in host gastric epithelial cell invasion and in modulation of matrix metalloproteinase 10 (MMP-10)

In Part I.1 of the Results, it was demonstrated that *H. pylori* infection induces host cell invasion that is dependent on the presence of a functional bacterial T4SS and is mediated by the c-Met receptor and its downstream targets Nck, PLC-γ, and c-Src.

Giving the fact that *H. pylori* virulence factors differentially interfere with signaling pathways in the host gastric epithelial cells, the role of CagA, of the T4SS, and of VacA in the induction of host cell invasion was studied. The experiments with different *H. pylori* strains clearly showed that maximal stimulation of cell invasion was achieved only when the bacteria have a functional T4SS. The presence of the T4SS could induce cell invasion either by the establishment of close contact with the host cell membrane, or by functioning as a translocator of an effector molecule. CagA is translocated by the T4SS (Odenbreit *et al.,* 2000) and, after injection into the host cell is able to modulate cellular functions by interacting intracellularly with the c-Met receptor and triggering morphological changes and motility similar to those induced by HGF (Segal *et al.*, 1999, Churin *et al.*, 2003, Al-Ghoul *et al.*, 2004). The observation that the number of invading cells after infection with a *cagA* mutant is lower than that observed with the wild type strain, suggests that CagA is important for stimulation of the invasive phenotype. These observations are in accordance with data showing that kidney canine cells transfected with *cagA* display an invasive phenotype (Bagnoli *et al.*, 2005). The fact that the T4SS is more critical than CagA for the induction of cell invasion suggests that additional effector molecule(s) might be translocated into the host cell via the T4SS. This is in accordance with previous published works showing that *H. pylori* can activate a number of signaling pathways in a T4SS-dependent, but CagA-independent manner (Crabtree *et al.*, 1995, Naumann *et al.*, 1999, El-Etr *et al.*, 2004). Additionally, it has also been shown that the T4SS is responsible for delivery of soluble compounds of peptidoglycan into the host cells (Viala *et al.*, 2004). In contrast to what was observed for the CagA and the T4SS

mutants, the VacA mutant induced similar levels of invasion to those of the wild-type strain, indicating that VacA is not involved in *H. pylori*-mediated cell invasion.

The c-Met receptor has been previously associated with invasion of cancer cells (Weidner *et al.*, 1990, Giordano *et al.*, 1992, Trusolino *et al.*, 2001). Furthermore, c-Met was also demonstrated to be a target of *H. pylori* CagA that is able to induce phosphorylation of the receptor, enhancing the cell motogenic response (Churin *et al.*, 2003). Due to these facts, the c-Met function in bacteria-mediated cell invasion was studied. The confirmation of the involvement of c-Met was demonstrated by treating the cells with the NK4 inhibitor or by transfecting the cells with siRNA directed to c-Met. In these conditions, *H. pylori* was not able to stimulate cell invasion, thus confirming the importance of c-Met on this cell phenotype.

The c-Met receptor is activated by *H. pylori* in a ligand-independent manner, resulting in host cell invasion. This fact is particularly relevant as a similar situation is described in gastric carcinoma patients where c-Met mutations (Chen *et al.*, 2001) or overexpression (Inoue *et al.*, 2004), result in the receptor activation in a ligand independent way. Interesting enough is also the fact that, in gastric carcinoma patients, the presence of c-Met has a significant association with poor prognosis and peritoneal dissemination (Toiyama *et al.*, 2012). It is also known that c-Met has a pro-metastatic and pro-motility activity mediated by reactive oxygen species (Ferraro *et al.*, 2006, Jagadeeswaran *et al.*, 2007). This fact should not be underestimated in the *H. pylori* infection context where there is an enhancement in the production of reactive oxygen and nitrogen species (Ding *et al.*, 2007, Elfvin *et al.*, 2007, Katsurahara *et al.*, 2009).

Upon binding of HGF, c-Met undergoes conformational changes leading to phosphorylation of specific tyrosine residues at the receptor intracellular domains, which act as docking sites for adaptor proteins and signal transducers. Once established that c-Met was involved in *H. pylori*-mediated cell invasion, the understanding of its downstream molecular mechanisms was the next step taken. Using a siRNA based strategy, a panel of c-Met adaptor molecules such as c-Cbl, Gab1, Nck, and Shc (Pelicci *et al.*, 1995, Kochhar *et al.*, 1996, Weidner *et al.*, 1996, Garcia-Guzman *et al.*, 2000), and other known downstream targets such as FAK, PLCγ, Shp-2, and c-Src (Okano *et al.*, 1993, Rahimi *et al.*, 1998, Beviglia *et al.*, 1999, Kodama *et al.*, 2000) were studied.

The silencing of Nck, PLC-γ, and c-Src impaired *H. pylori*-mediated cell invasion in a significant manner. PLC-γ was previously reported to be activated after *H. pylori* infection (Churin *et al.*, 2003, Franke *et al.*, 2008). The involvement of this enzyme in the induction of host cell invasion by *H. pylori* is in agreement with previous results showing its involvement in the motogenic response induced by the bacteria (Churin *et al.*, 2003), a

condition required for a cell to become invasive. In this line, and in a non-infectious context, there are descriptions showing that PLC-γ is also linked to cytoskeletal alterations needed to cell motility (Wells *et al.*, 1999, Jones *et al.*, 2005).

In this Part I.1 of Results, and for the first time, a role for Nck in the context of *H. pylori* infection was described. In another infection model, it has been shown that the EPEC virulence factor Tir is able to interact with Nck, inducing actin pedestal formation necessary for bacteria internalization into the host cell (Gruenheid *et al.*, 2001). In a non-infectious context, it has also been described that Nck functions downstream the RTK PDGFR (Rivera *et al.*, 2006, Ruusala *et al.*, 2008, Abella *et al.*, 2010). Nck is involved in cytoskeletal re-organization, cell motility (Rohatgi *et al.,* 2001; Zhou *et al.,* 2003; Rivera *et al.,* 2004; Rivera *et al.,* 2009), and in invadopodia formation (Stylli *et al.*, 2009, Oser *et al.*). All these cellular processes are important for cell invasion, which is in accordance with the results obtained in the case of *H. pylori* infection.

c-Src kinase is involved in the induction of cellular alterations by *H. pylori,* working as a CagA kinase (Selbach *et al.*, 2002). In addition, c-Src is involved in the process of cancer invasion in non-gastric models (Chiang *et al.*, 2005, Herynk *et al.*, 2007, Lawler *et al.*, 2009). c-Src appears to mediate cancer invasion via c-Met activation (Herynk *et al.*, 2007), or by stimulation with inflammatory cytokines (Lin *et al.*, 2007). The discovery of the involvement of c-Src in *H. pylori*-mediated cell invasion is, therefore, not surprising.

The downregulation of FAK also resulted in decreased cell invasion, though not in a significant manner. However, a role for FAK kinase in the induction of an invasive phenotype by the bacteria can not be excluded. The involvement of FAK in alterations induced by *H. pylori* has been previously described (Pai *et al.*, 1999, Tsutsumi *et al.*, 2006, Tabassam *et al.*, 2008, Tabassam *et al.*, 2011, Tegtmeyer *et al.*, 2011). Additionally, it has been shown that FAK can function downstream of c-Met, mediating alterations in cell motility and invasion in other systems (Chen *et al.*, 2006, Hui *et al.*, 2009).

The observations that Nck, PLC-γ, and c-Src are activated downstream of c-Met are in accordance with previous reports (Okano *et al.*, 1993, Rahimi *et al.*, 1998, Beviglia *et al.*, 1999, Kodama *et al.*, 2000, Kochhar *et al.*, 1996). Data in the literature, together with the findings obtained in this Part I.1 of the Results, point to the importance of RTK-mediated signaling in host cell alterations induced by *H. pylori*.

Cell invasion has a proteolytic component in which metalloproteinases are key enzymes. MMPs are able to degrade components of the extracellular matrix, process cytokines and growth factors, and cleave cell surface receptors that act as signal transducers in invasion pathways (Egeblad *et al.*, 2002).

In the context of *H. pylori* infection, it was already known that the presence of bacteria enhance the production and activation of MMPs in epithelial cells, contributing to gastric tissue damage (Bebb *et al.*, 2003, Crawford *et al.*, 2003, Gooz *et al.*, 2003, Wroblewski *et al.*, 2003, Krueger *et al.*, 2006). The molecular mechanisms triggered by *H. pylori* leading to up-regulation of MMPs have however not been fully explored.

In Part I.2 of the Results of this thesis, it was also validated that *H. pylori* leads to an increase in the expression of MMP-1, MMP-7, and MMP-10 in the AGS cell infection model system. These results confirmed the reports showing that *H. pylori* infection up-regulates MMP-1 (Kitadai *et al.*, 2003, Krueger *et al.*, 2006, Pillinger *et al.*, 2007) and MMP-7 (Bebb *et al.*, 2003, Crawford *et al.*, 2003, Gooz *et al.*, 2003, Wroblewski *et al.*, 2003, Krueger *et al.*, 2006). MMP-10 has gained attention during the last years due to its association with a broad spectrum of pathological conditions, such as cancer and inflammatory and cardiovascular diseases (Gill *et al.*, 2004, P *et al.*, 2001, Mathew *et al.*, 2002, Thorns *et al.*, 2003, Bodey *et al.*, 2000, Saghizadeh *et al.*, 2001, Ogata *et al.*, 2005). Increased expression of MMP-10 has been described in gastric carcinoma tissue (Aung *et al.*, 2006, Lee *et al.*, 2009). Furthermore, cDNA microarray studies to investigate changes in gene expression in hypergastrinemic transgenic (INS-GAS) mice stomachs infected with *H. felis* (Takaishi *et al.*, 2007), and in a mouse gastric epithelial progenitor cell line infected with *H. pylori* (Giannakis *et al.*, 2008), have suggested that *Helicobacter* up-regulates MMP-10. In this thesis, and for the first time, the role of *H. pylori* infection on the modulation of MMP-10 expression, as well as the regulation of this process in human gastric epithelial cells, was explored.

It was demonstrated that infection with pathogenic *H. pylori* strains, such as 26695 and 84183, increase MMP-10 mRNA, protein secretion and activity. The experiments showed that *H. pylori* is able to increase MMP-10 mRNA levels as high as 40-fold compared with non-infected conditions. These expression levels are much higher than the ones reported in an expression array comparing infected and non-infected cells (Krueger *et al.*, 2006), and this difference may be due to the use of a different strain and a different MOI.

That MMP-10 up-regulation is associated with CagA-positive strains was demonstrated by results obtained with infection of AGS cells with a *H. pylori cagA*-mutant. These results were further validated using a panel of *H. pylori* clinical isolates with known *cagA* status. Infection with strains that are more virulent such as CagA-positive strains, results in more severe disease, such as atrophic gastritis, peptic ulcer disease, and gastric carcinoma (Crabtree *et al.*, 1991b, Figueiredo *et al.*, 2002, Blaser *et al.*, 1995, Peek *et al.*, 1995). One can speculate that *H. pylori* CagA-positive strains lead to high levels of MMP-10

expression and protein secretion, leading to aberrant signaling, making the individuals infected with such strains at higher risk of disease.

In a study where normal versus gastric carcinoma tissues were analyzed with a SAGE approach, and where MMP-10 was identified as a cancer associated protein, 15 out of 20 serum samples of gastritis patients did not present MMP-10 expression. In that study, however, the *H. pylori* status was not determined (Aung *et al.*, 2006). This may be an indication that MMP-10 does not have a role in gastritis associated with *H. pylori* colonization, but may be associated with more severe disease.

A role for MMP-10 in cell invasion in *H. pylori* infection context was also demonstrated for the first time. Cells with abrogation of MMP-10 expression and infected with *H. pylori* were significantly less invasive than non-silenced infected cells. Previous reports in non-infectious contexts showed that MMP-10 expression is associated with increase invasion of carcinoma cells (Frederick *et al.,* 2008, Deraz *et al.,* 2011).

The results that c-Met and EGFR are associated with *H. pylori*-mediated MMP-10 expression are novel findings. It has been shown that MMPs may be up-regulated by growth factors that activate RTKs (McCawley *et al.*, 2001, Egeblad *et al.*, 2002). c-Met and EGFR are RTKs previously reported to be targeted by *H. pylori* (Wallasch *et al.*, 2002, Churin *et al.*, 2003). In fact, c-Met is one of the CagA targets (Churin *et al.*, 2003, Oliveira *et al.*, 2006, Oliveira *et al.*, 2009), in keeping with our results that CagA-positive strains are significantly better inducers of MMP-10 than CagA-negative strains.

The EGFR was previously shown to be involved in MMP-10 up-regulation in squamous cell carcinoma of head and neck (Wilkins-Port *et al.*, 2007). Moreover, and in the context of *H. pylori* infection, EGF family members were shown to be involved in the stimulation of other MMPs, such as MMP-7, (Wroblewski *et al.*, 2003). On the other hand, *H. pylori*-mediated MMP up-regulation results in an increase of HB-EGF processing, a ligand of EGFR, and thus leads to an increase in the receptor activation (Wallasch *et al.*, 2002, Yin *et al.*, 2010). Although apparently contradicting, these data may point to a feedback regulation mechanism mediated by MMPs during *H. pylori* infection. The fact that two RTKs are involved in the same cellular process may be an indication that they cooperate and compensate the absence of the other. This hypothesis is in accordance with previous descriptions of cross-talk between c-Met and EGFR (Jo *et al.*, 2000, Nath *et al.*, 2001, Xu *et al.*, 2007, Mueller *et al.*, 2008).

Of the molecules involved in *H. pylori*-mediated cell invasion described in Part I.1 of the Results, Nck, PLC-γ, and c-Src, all described to function downstream c-Met and EGFR (Okano *et al.*, 1993, Chang *et al.*, 1995, Kochhar *et al.*, 1996, Rahimi *et al.*, 1998, Kassis *et al.*, 1999, Hake *et al.*, 2008), only Nck and c-Src were shown to be involved

in MMP-10 modulation after infection. Giving that Nck is an adaptor protein of the RTKs studied, it may function in the early stages of signaling transduction. The role of c-Src is not surprising since this is a kinase of CagA, a virulence factor that is essential to MMP-10 increased expression. Whether the phosphorylation of CagA is necessary to MMP-10 up-regulation will be an interesting issue to address. Indeed, in a previous report the inhibition of CagA phosphorylation using a c-Src chemical inhibitor resulted in a decrease of *H. pylori*-mediated MMP-9 secretion (Nam *et al.*, 2011).

Our Group has previously shown that *H. pylori* T4SS-competent and CagA-positive strains induce AGS cell invasion via c-Met receptor activation and increased activities of MMP-2 and MMP-9 (Oliveira *et al.*, 2006). It is plausible that phosphorylation of tyrosine residues at the c-Met receptor intracellular domains mediated by CagA results in the phosphorylation and binding of adaptor proteins and activation of signal transducers, including Nck and c-Src, eventually leading to MMP-10 expression and cell invasion.

Our Group has previously observed that the increase in the proteolytic activities of MMP-2 and MMP-9 upon *H. pylori* infection, significantly decreased after silencing c-Met expression, suggesting that c-Met is involved in the activation of these MMPs (Oliveira *et al.*, 2006). Adding to this observation, it has been shown that MMP-10 cleaves and activates pro-MMP-9 (Nakamura *et al.*, 1998). Whether c-Met-mediated MMP-10 expression plays a role in pro-MMP-9 activation in the context of *H. pylori* infection will be an interesting topic of further studies.

It was demonstrated that the ERK1/2 and JNK signaling pathways are involved in *H. pylori*-induced MMP-10 expression. These observations, together with the finding that EGFR is involved in *H. pylori* stimulation of MMP-10 are also consistent with previous reports of *H. pylori*-mediated ERK activation via transactivation of the EGFR (Keates *et al.*, 2001, Keates *et al.*, 2005). The involvement ERK1/2, JNK, and p38 signal transduction pathways in MMP regulation has been previously described (Reunanen *et al.*, 1998, Ridley *et al.*, 1997), including in the context of *H. pylori* infection (Crawford *et al.*, 2003, Krueger *et al.*, 2006, Pillinger *et al.*, 2007, Nam *et al.*, 2011). In contrast to ERK and JNK, our results showed that inhibition of p38 signaling significantly stimulated MMP-10 expression. Curiously, the finding that inhibition of p38 enhanced MMP-10 expression was also reported in head and neck cancer (Deraz *et al.*, 2011). Our results with *H. pylori* infection are similar to those obtained by Pillinger *et al.* using the same AGS cell line, and which detected a significantly enhancing effect of the p38 inhibitor SB203580 on MMP-1 expression (Pillinger *et al.*, 2007). Similar findings were also obtained in AGS cells by Nam *et al*. in which p38 inhibitor slightly activated MMP-9 secretion (Nam *et al.*, 2011).

A panel of cytokines associated to *H. pylori* infection, including IL-1β, TNF-α, INF-γ, TGF-β, IL-10, and IL-8 (Romero-Adrian *et al.*, 2010), were evaluated in the modulation of MMP-10 expression. Of these, only IL-1β was able to induce an overexpression of MMP-10. This is particularly interesting giving the previously described role of IL-1β during *H. pylori* infection in increasing gastric cell secretion of MMP-3 (Gooz *et al.*, 2003), a MMP of the same sub-family of MMP-10.

The results obtained here suggest a role for MMP-10 during *H. pylori* infection in the activation of MMP-1. However, that is probably not the only mechanism of MMP-1 activation, since the silencing of MMP-10 was not sufficient to abrogate active MMP-1 present in conditioned medium. This is particularly interesting because MMPs are regulated at several levels, both transcriptional and post-transcriptional. One of the post-transcriptional regulatory events is the activation of the pro-enzyme to an active form, which is probably what occurs in MMP-1 regulation by MMP-10. While previous works that described an association between MMP-1 up-regulation and *H. pylori* infection have focused on the transcriptional regulation (Krueger *et al.*, 2006, Pillinger *et al.*, 2007, Sokolova *et al.*, 2012), this is the first study describing a post-transcriptional process leading to MMP-1 activation. The fact that MMP-10 may function as activator of other MMPs, makes this metalloproteinase a special player in the amplification of the general MMP response during *H. pylori* infection.

The studies associating the up-regulation of MMPs to infection with *H. pylori* usually describe the final result of MMP activation as effectors of invasion and migration. However, MMPs are also molecular regulators able to modulate a plethora of chemokines, reactive oxygen species, and growth factors that, in turn, act in the induction of several pathological processes (Parks *et al.*, 2004)**.** Future studies addressing the role of MMPs during *H. pylori* infection not only as modulators of tissue damage, but also as modulators of other pathological processes, should be of critical importance. The increase in production of MMPs associated to infection with *H. pylori* virulent strains may account for gastric tissue damage and for the ability of host epithelial cells to invade surrounding tissues. This may also suggest a role for the bacterium in later stages of gastric carcinogenesis.

In conclusion, these results show that MMP-10 expression is induced in gastric epithelial cells by CagA-positive *H. pylori* strains *via* the c-Met and EGF receptors, in a process that involves Nck and Src, and also the ERK and JNK pathways. Our results also demonstrate that MMP-10 is involved in *H. pylori*-mediated gastric cell invasion (Figure 33). In the future, it will be interesting to address whether targeting MMPs in the context

of *H. pylori* infection, either by direct inhibitors or by targeting the signaling pathways that up-regulate MMP expression, will improve the outcome of the infection.

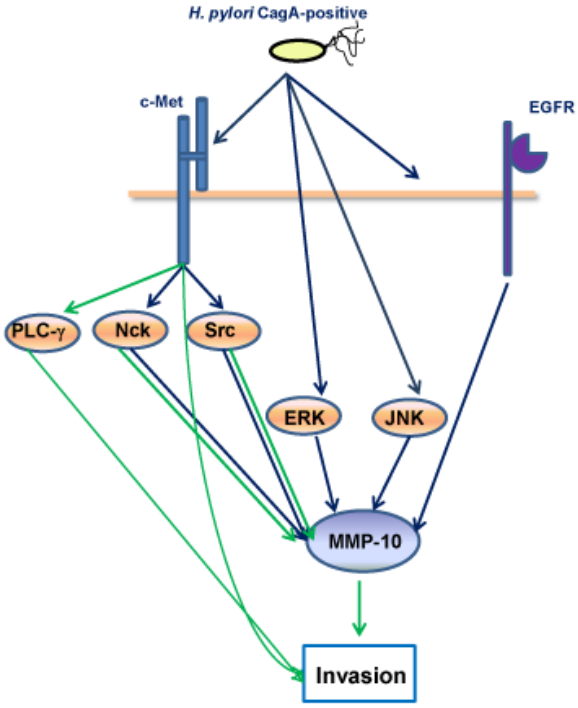


Figure 33. *H. pylori-mediated cell invasion and MMP-10 up-regulation. H. pylori* CagA-positive strains induce gastric cell invasion via c-Met-mediated PLC-γ, Nck, and c-Src activation. MMP-10 is up-regulated by *H. pylori* cagA-positive strains via the c-Met and EGFR, in a process that involves Nck and c-Src, and also ERK and JNK pathways. MMP-10 is also an important molecule for cell invasion induced by *H. pylori.* Green arrows refer to findings reported in this thesis regarding *H. pylori*-mediated cell invasion, and dark blue arrows refer to results regarding MMP-10 up-regulation.

Part II. Analysis of the effects of E-cadherin in *H. pylori*-mediated cell invasion and signaling

In Part II of the Results, it was demonstrated that *H. pylori* alters the E-cadherin/catenin complex, leading to the formation of a multiproteic complex composed of CagA, c-Met, E-cadherin, and p120-catenin. Due to the establishment of this complex, *H. pylori*-induced c-Met and p120-catenin phosphorylation as well as *H. pylori*-induced cell invasive phenotype were abrogated.

In cell lines containing an intact E-cadherin/catenin complex, *H. pylori* induced lower

levels or no invasion in comparison with AGS cells. Using two clones from an E-cadherin stably transduced cell line (AGSEcad), in which a functional E-cadherin/catenin complex was established, it was shown that E-cadherin was sufficient to suppress the invasive phenotype induced by *H. pylori*.

Induction of AGS cell invasion by *H. pylori* involves c-Met tyrosine phosphorylation (Oliveira *et al.*, 2006). In contrast, *H. pylori* decreases the phosphorylation levels of c-Met in cells with a stable E-cadherin/catenin complex. Also, depending on the E-cadherin status, infection with *H. pylori* differently affects the tyrosine phosphorylation of p120-catenin. In cells lacking E-cadherin expression, *H. pylori* induced p120-catenin phosphorylation, whereas in cells expressing E-cadherin *H. pylori* led to reduced p120-catenin phosphorylation. Taken together, these data point to a role of E-cadherin in the suppression of *H. pylori*-induced c-Met and p120-catenin phosphorylation and, consequently, in the suppression of the cell invasive phenotype.

As already mentioned, c-Met is a receptor tyrosine kinase with a well-documented participation in cell invasion. p120-catenin interacts with the cytoplasmic juxtamembrane domain of E-cadherin (Yap *et al.*, 1998), which, in conjunction with the phosphorylation status of p120-catenin, plays an important role in the stabilization of E-cadherin (Reynolds *et al.*, 2004a, Yanagisawa *et al.*, 2006). Phosphorylation of p120-catenin promotes its dissociation from E-cadherin and its translocation to the cytoplasm or nucleus where it participates in many signaling events (van Hengel *et al.*, 1999). Endogenous p120-catenin is described to promote migration and invasiveness of E-cadherin-deficient cells (Yanagisawa *et al.*, 2006). In agreement with these findings are the ones performed in experimental models, where re-establishment of E-cadherin function in E-cadherin-deficient cell lines reversed the invasive phenotype, pointing to E-cadherin role in suppression of cell invasion (Frixen *et al.*, 1991, Vleminckx *et al.*, 1991, Suriano *et al.*, 2003a).

The phosphorylation statuses of c-Met and of p120-catenin are regulated by kinases and phosphatases such as c-Src, Fer, Shp-2, and PTP1B (Reynolds *et al.*, 1989, Kim *et al.*, 1995, Balsamo *et al.*, 1996, Ukropec *et al.*, 2000). Because differences in cell invasion are associated with differences in c-Met and p120-catenin phosphorylation, a potential role for these kinases and phosphatases in the process of invasion is an interesting issue to explore.

In both AGSEcad and NCI-N87 cells, E-cadherin expression levels were not altered by *H. pylori,* which is in accordance with previously published data in *in vitro* models (Conlin *et al.*, 2004, Bebb *et al.*, 2006). Although there is one description of an association between *H. pylori* infection and downregulation of E-cadherin expression (Terres *et al.*, 1998), the

findings presented in Part II of the Results of this thesis are also in accordance with those of the majority of the studies using gastric biopsy specimens that show no association between the two events (Bebb *et al.*, 2006, Zullo *et al.*, 2004, Shun *et al.*, 2001, Chan *et al.*, 2003b). In line with the observations of others, it was observed that *H. pylori* infection also did not affect the expression levels of p120-, β-, or α-catenins (Bebb *et al.*, 2006, Krueger *et al.*, 2007). Although no differences were observed in the expression of these molecules, *H. pylori* infection led to increased intensity in membrane staining of E-cadherin and of p120-catenin. These were also the experimental evidences from the immunoprecipitation studies, showing enhanced E-cadherin/p120-catenin binding after infection, suggesting that *H. pylori* alters the organization of the E-cadherin/catenin complex.

After injection into the host cell cytoplasm the CagA T4SS effector localizes to the inner surface of the plasma membrane (Higashi *et al.*, 2005). The confocal immunostainings pointed to co-localization of CagA and E-cadherin, and, most interestingly, to co-localization of CagA and p120-catenin at the cell membrane. The immunoprecipitation assays using *in vitro* infection revealed that CagA indeed physically interacts with E-cadherin, with c-Met, and with p120-catenin. The finding that CagA interacts with E-cadherin is in agreement with the work of Murata-Kamiya *et al.* who reported that in CagA-transfected cells, CagA interacts with E-cadherin, destabilizing the E-cadherin/β-catenin binding (Murata-Kamiya *et al.*, 2007). The findings reported in this thesis also confirm previous observations of the interaction between CagA and c-Met (Churin *et al.*, 2003), and add novelty to the field of study by showing interaction between CagA and p120-catenin.

Another interesting observation in this study was that after *H. pylori* infection, c-Met/E-cadherin, and c-Met/p120-catenin interactions were enhanced. Taken together with the CagA interactions results, these observations suggest that CagA, E-cadherin, p120-catenin, and c-Met interact with each other possibly forming a multiproteic complex. Furthermore, experiments with siRNA targeting c-Met revealed that, in the absence of c-Met, CagA was no longer able to interact with E-cadherin or with p120-catenin, suggesting that interactions of CagA with the two elements of the E-cadherin/catenin complex occur via c-Met.

The hypothesis formulated taking these results into consideration is that after infection, injected CagA interacts with c-Met and this complex now interacts with the E-cadherin/catenin complex, leading to the formation of a multiproteic complex (Figure 34). This model is supported by the co-immunoprecipitations and siRNA results and might explain the reorganization of the E-cadherin/catenin complex observed by

immunocytochemistry. Further studies should be performed to elucidate how these four molecules interact within the multiproteic complex. There is now increasing evidence that E-cadherin participates in and regulates several signaling pathways via its extracellular domain (McLachlan *et al.*, 2007). The extracellular domain of E-cadherin establishes not only homophilic, but also heterophilic interactions with tyrosine kinase receptors such as c-Met (Hiscox *et al.*, 1999, Mateus *et al.*, 2007, Reshetnikova *et al.*, 2007). It is possible that the c-Met/E-cadherin interaction is established via their extracellular domain and that *H. pylori* CagA interacts with both E-cadherin and p120-catenin via c-Met.

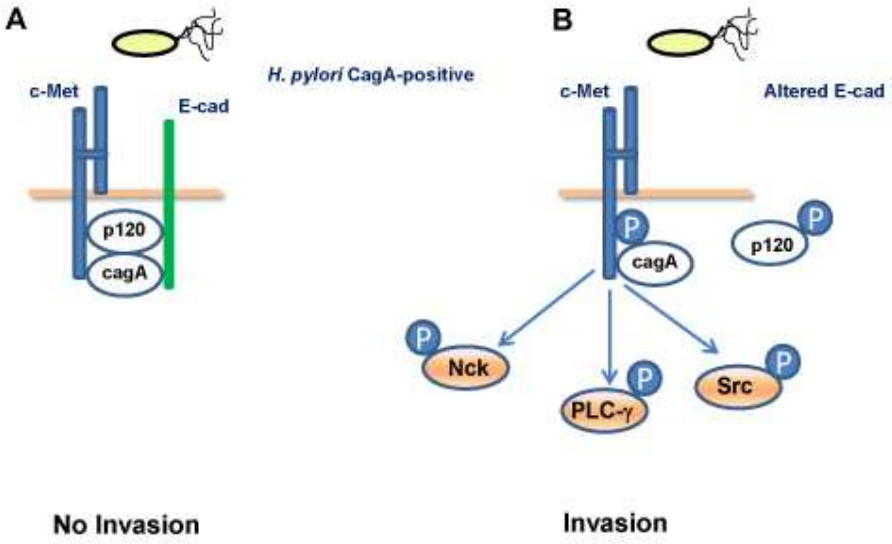


Figure 34. *H. pylori and host cell invasion.* **(A)** In cells with an intact E-cadherin/catenin complex *H. pylori* infection leads to enhanced c-Met/E-cadherin, c-Met/p120-catenin, and E-cadherin/p120-catenin interactions. In these cells, CagA binds to c-Met and this complex now interacts with the E-cadherin/catenin complex, suppressing *H. pylori*-mediated c-Met and p120-catenin phosphorylation and cell invasive phenotype **(B)** In E-cadherin negative cells, *H. pylori* CagA leads to an increase in the phosphorylation levels of both p120-catenin and c-Met. The activation of c-Met and its downstream targets Nck, c-Src, and PLC-γ results in host cell invasion.

Disruption of the adherens junctions by *H. pylori* has been shown in several reports using cell line models (Conlin *et al.*, 2004, Weydig *et al.*, 2007, Schirrmeister *et al.*, 2009, Hoy *et al.*, 2010). These alterations may involve E-cadherin shedding via ADAM-10 induction by *H. pylori* or via the secreted bacterial protease HtrA (Schirrmeister *et al.*, 2009, Hoy *et al.*, 2010). Furthermore, it has been shown that *H. pylori* induces translocation of E-cadherin from the cell membrane to intracellular location (Conlin *et al.*, 2004, Weydig *et al.*, 2007, Schirrmeister *et al.*, 2009). In both cases, E-cadherin alterations induced by *H. pylori*

result in phenotypic changes such as cell scattering and elongation, increased cell migration and invasion. The apparent discrepancies between these observations and the results obtained in this thesis may derive from differences in infection time and cell lines used. Here, the phosphorylation and co-immunoprecipitation assays were performed 1 hour after infection, a short-term infection time, and it is possible that longer infection periods result in different phenotypes. Nevertheless, the invasion assays were performed after 24 hours of infection, and corroborate the results obtained after 1 hour infection, showing that the potential inactivation of the c-Met due to its interaction with E-cadherin suppresses the invasive phenotype induced by the bacteria.

It should also be noted that when one is addressing the issue of *H. pylori* infection in cell-cell junction dysfunction two main aspects should be taken into consideration. First, a lot of studies have been performed in cell line models, often non-human and non-gastric, and frequently cancer-derived and with impaired cell-cell junctions. For example, the AGS and the MKN45 cell lines harbor mutations in the E-cadherin gene (Yokozaki, 2000). Also, genetic alterations in components of the Wnt signaling pathway are common in cell lines such as MKN7, MKN28 and HT29 (Yokozaki, 2000, Hsi *et al.*, 1999). In addition, in host cell lines and bacteria co-culture models, infections cannot easily be maintained for long time periods, making these models more close to acute that to chronic infections. Second, results obtained in cell lines do not match those observed in the gastric mucosa of *H. pylori*-infected patients. In particular, gastric biopsy specimens of patients with and without *H. pylori* infection do not show any relationship between the infection and changes in the expression or distribution of E-cadherin, β-catenin, or p120-catenin. This does not mean that the phenomena observed *in vitro* do not occur *in vivo,* but may be explained if *H. pylori*-induced alterations in the adherens junctions are fast and transient and, therefore, not observable in fixed gastric tissues. Another possible explanation is that alterations in adherens junctions induced by *H. pylori* only occur in a subset of cells. These cells may then be selected as having growth advantages or increased resistance to apoptosis, therefore rendering them more susceptible to accumulation of DNA lesions which may lead to malignant transformation.

Concluding remarks

It is possible to make an analogy between what occurs during infection with *H. pylori* and the exploitation of the natural resources by the human being. In order to obtain cheap

food and raw materials to overcome its needs, Man can damage its ecosystem in an irreversible way, which can compromise the future viability of the species in the habitat. In a similar way, *H. pylori* infection, since it is chronic and acquired during childhood persisting through the host's lifetime, may induce, in the long term, such a pressure in the ecosystem that compromises either the viability of the gastric tissue, or bacterial survival. Since we are talking about an ecosystem, it is important to stress that changes in one particular process may affect all the other processes of the system in a way that sometimes may not be very clear. Therefore, any particular molecular process should be understood as being part of something bigger, and the final phenotype observed may be understood as the interplay of several variables.

It is known that host, bacterium, and environmental factors account for the differences in the clinical outcome of *H. pylori* infection. Although it is known that only a small proportion of infected individuals develop gastric carcinoma, the exact mechanisms underlying disease development mediated by *H. pylori* infection remain unraveled. The interaction between *H. pylori* and gastric epithelial cells leads to activation of host signaling pathways, modification of cellular functions, and induction of cell phenotypes important for carcinogenesis.

Results in this thesis suggest that in cells with intact adherens junctions *H. pylori* leads to formation of a multiproteic complex composed of CagA, c-Met, E-cadherin, and p120-catenin which impairs c-Met and p120-catenin tyrosine phosphorylation and suppresses cell invasion induced by *H. pylori*. In contrast, in the absence of E-cadherin, *H. pylori* infection associates with an increased ability of the cells to invade. It is arguable whether the invasive phenotype observed in the Matrigel *in vitro* cell model overlaps with tumor cell invasion, as observed during tumor progression *in vivo*. The latter is the result of a multistage process involving other events in addition to E-cadherin impairment; in fact it is arguable whether *H. pylori* would play a role in tumor invasion, because often, *H. pylori* is not present in full-blown gastric carcinoma. Instead, *H. pylori* infection might play an important role in selecting for survival gastric epithelial cells harboring E-cadherin inactivation (genetic or epigenetic) that would otherwise undergo apoptosis before actual tumor progression occurs. In fact, recent data showed that cells lacking a functional E-cadherin have increased survival when compared to E-cadherin wild-type cells (Ferreira *et al.*, 2012). In other words, E-cadherin inactivation is an initiator event, whereas *H. pylori* infection works as a tumor promoter. As such, *H. pylori* infection would increase the likelihood of E-cadherin-deficient cell lineage survival and further accumulation of oncogenic events in a process that, in the early phase, becomes *H. pylori* independent.

REFERENCES

Abella, J.V., Vaillancourt, R., Frigault, M.M., Ponzo, M.G., Zuo, D., Sangwan, V.*, et al.* (2010). The Gab1 scaffold regulates RTK-dependent dorsal ruffle formation through the adaptor Nck. *J Cell Sci* **123,** 1306-1319.

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J* **16,** 3797-3804.

Al-Ghoul, L., Wessler, S., Hundertmark, T., Kruger, S., Fischer, W., Wunder, C.*, et al.* (2004). Analysis of the type IV secretion system-dependent cell motility of Helicobacter pylori-infected epithelial cells. *Biochem Biophys Res Commun* **322,** 860-866.

Amemiya, H., Kono, K., Mori, Y., Takahashi, A., Ichihara, F., Iizuka, H.*, et al.* (2000). High frequency of c-Met expression in gastric cancers producing alpha- fetoprotein. *Oncology* **59,** 145-151.

Amieva, M.R., Vogelmann, R., Covacci, A., Tompkins, L.S., Nelson, W.J. and Falkow, S. (2003). Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. *Science* **300,** 1430-1434.

Asahi, M., Tanaka, Y., Izumi, T., Ito, Y., Naiki, H., Kersulyte, D.*, et al.* (2003). Helicobacter pylori CagA containing ITAM-like sequences localized to lipid rafts negatively regulates VacA-induced signaling in vivo. *Helicobacter* **8,** 1-14.

Ashktorab, H., Daremipouran, M., Wilson, M., Siddiqi, S., Lee, E.L., Rakhshani, N.*, et al.* (2007). Transactivation of the EGFR by AP-1 is induced by Helicobacter pylori in gastric cancer. *Am J Gastroenterol* **102,** 2135-2146.

Atherton, J.C., Cao, P., Peek, R.M., Jr., Tummuru, M.K., Blaser, M.J. and Cover, T.L. (1995). Mosaicism in vacuolating cytotoxin alleles of Helicobacter pylori. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* **270,** 17771-17777.

Atherton, J.C., Tham, K.T., Peek, R.M., Jr., Cover, T.L. and Blaser, M.J. (1996). Density of Helicobacter pylori infection in vivo as assessed by quantitative culture and histology. *J Infect Dis* **174,** 552-556.

Aung, P.P., Oue, N., Mitani, Y., Nakayama, H., Yoshida, K., Noguchi, T.*, et al.* (2006). Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. *Oncogene* **25,** 2546-2557.

Azevedo, N.F., Guimaraes, N., Figueiredo, C., Keevil, C.W. and Vieira, M.J. (2007). A new model for the transmission of Helicobacter pylori: role of environmental reservoirs as gene pools to increase strain diversity. *Critical reviews in microbiology* **33,** 157-169.

Bagnoli, F., Buti, L., Tompkins, L., Covacci, A. and Amieva, M.R. (2005). Helicobacter pylori CagA induces a transition from polarized to invasive phenotypes in MDCK cells. *Proc Natl Acad Sci U S A* **102,** 16339-16344.

Balda, M.S. and Matter, K. (2008). Tight junctions at a glance. *J Cell Sci* **121,** 3677-3682.

Balsamo, J., Leung, T., Ernst, H., Zanin, M.K., Hoffman, S. and Lilien, J. (1996). Regulated binding of PTP1B-like phosphatase to N-cadherin: control of cadherin-mediated adhesion by dephosphorylation of beta-catenin. *J Cell Biol* **134,** 801-813.

Bardelli, A., Longati, P., Gramaglia, D., Basilico, C., Tamagnone, L., Giordano, S.*, et al.* (1998). Uncoupling signal transducers from oncogenic MET mutants abrogates cell transformation and inhibits invasive growth. *Proc Natl Acad Sci U S A* **95,** 14379-14383.

Basso, D., Zambon, C.F., Letley, D.P., Stranges, A., Marchet, A., Rhead, J.L.*, et al.* (2008). Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. *Gastroenterology* **135,** 91-99.

Basu, S., Pathak, S.K., Chatterjee, G., Pathak, S., Basu, J. and Kundu, M. (2008). Helicobacter pylori protein HP0175 transactivates epidermal growth factor receptor through TLR4 in gastric epithelial cells. *J Biol Chem* **283,** 32369-32376.

Bauer, B., Bartfeld, S. and Meyer, T.F. (2009). H. pylori selectively blocks EGFR endocytosis via the non-receptor kinase c-Abl and CagA. *Cell Microbiol* **11,** 156-169.

Bebb, J.R., Leach, L., Zaitoun, A., Hand, N., Letley, D.P., Thomas, R. and Atherton, J.C. (2006). Effects of Helicobacter pylori on the cadherin-catenin complex. *J Clin Pathol* **59,** 1261-1266.

Bebb, J.R., Letley, D.P., Thomas, R.J., Aviles, F., Collins, H.M., Watson, S.A.*, et al.* (2003). Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner. *Gut* **52,** 1408-1413.

Behrens, J., Vakaet, L., Friis, R., Winterhager, E., Van Roy, F., Mareel, M.M. and Birchmeier, W. (1993). Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol* **120,** 757-766.

Benvenuti, S. and Comoglio, P.M. (2007). The MET receptor tyrosine kinase in invasion and metastasis. *Journal of cellular physiology* **213,** 316-325.

Bergin, P.J., Anders, E., Sicheng, W., Erik, J., Jennie, A., Hans, L*., et al.* (2004). Increased production of matrix metalloproteinases in Helicobacter pylori-associated human gastritis. *Helicobacter* **9,** 201-210.

Bergin, P.J., Sicheng, W., Qiang, P.H. and Marianne, Q.J. (2005). Secretion of matrix metalloproteinase-9 by macrophages, in vitro, in response to Helicobacter pylori. *FEMS immunology and medical microbiology* **45,** 159-169.

Beswick, E.J. and Reyes, V.E. (2008). Macrophage migration inhibitory factor and interleukin-8 produced by gastric epithelial cells during Helicobacter pylori exposure induce expression and activation of the epidermal growth factor receptor. *Infect Immun* **76,** 3233-3240.

Beviglia, L. and Kramer, R.H. (1999). HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells. *Int J Cancer* **83,** 640-649.

Bierie, B., Nozawa, M., Renou, J.P., Shillingford, J.M., Morgan, F., Oka, T*., et al.* (2003). Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation. *Oncogene* **22,** 3875-3887.

Bilder, D., Li, M. and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289,** 113-116.

Birchmeier, C., Birchmeier, W., Gherardi, E. and Vande Woude, G.F. (2003). Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4,** 915-925.

Blaser, M.J., Perez-Perez, G.I., Kleanthous, H., Cover, T.L., Peek, R.M., Chyou, P.H*., et al.* (1995). Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* **55,** 2111-2115.

Bodey, B., Bodey, B., Jr., Siegel, S.E. and Kaiser, H.E. (2000). Immunocytochemical detection of MMP-3 and -10 expression in hepatocellular carcinomas. *Anticancer Res* **20,** 4585-4590.

Borowiak, M., Garratt, A.N., Wustefeld, T., Strehle, M., Trautwein, C. and Birchmeier, C. (2004). Met provides essential signals for liver regeneration. *Proc Natl Acad Sci U S A* **101,** 10608-10613.

Boterberg, T., Vennekens, K.M., Thienpont, M., Mareel, M.M. and Bracke, M.E. (2000). Internalization of the E-cadherin/catenin complex and scattering of human mammary carcinoma cells MCF-7/AZ after treatment with conditioned medium from human skin squamous carcinoma cells COLO 16. *Cell adhesion and communication* **7,** 299-310.

Boughan, P.K., Argent, R.H., Body-Malapel, M., Park, J.H., Ewings, K.E., Bowie, A.G*., et al.* (2006). Nucleotide-binding oligomerization domain-1 and epidermal growth factor receptor: critical regulators of beta-defensins during Helicobacter pylori infection. *J Biol Chem* **281,** 11637-11648.

Brenes, F., Ruiz, B., Correa, P., Hunter, F., Rhamakrishnan, T., Fontham, E. and Shi, T.Y. (1993). Helicobacter pylori causes hyperproliferation of the gastric epithelium: pre- and post-eradication indices of proliferating cell nuclear antigen. *Am J Gastroenterol* **88,** 1870-1875.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler-Stevenson, W.G*., et al.* (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* **85,** 683-693.

Brunelleschi, S., Penengo, L., Santoro, M.M. and Gaudino, G. (2002). Receptor tyrosine kinases as target for anti-cancer therapy. *Current pharmaceutical design* **8,** 1959-1972.

Brusevold, I.J., Aasrum, M., Bryne, M. and Christoffersen, T. (2012). Migration induced by epidermal and hepatocyte growth factors in oral squamous carcinoma cells in vitro: role of MEK/ERK, p38 and PI-3 kinase/Akt. *J Oral Pathol Med*.

Caputo, R., Tuccillo, C., Manzo, B.A., Zarrilli, R., Tortora, G., Blanco Cdel, V*., et al.* (2003). Helicobacter pylori VacA toxin up-regulates vascular endothelial growth factor expression in MKN 28 gastric cells through an epidermal growth factor receptor-, cyclooxygenase-2-dependent mechanism. *Clin Cancer Res* **9,** 2015-2021.

Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. *Annual review of biochemistry* **56,** 881-914.

Carragher, N.O. and Frame, M.C. (2004). Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends in cell biology* **14,** 241-249.

Caruso, R., Fina, D., Peluso, I., Fantini, M.C., Tosti, C., Del Vecchio Blanco, G.*, et al.* (2007). IL-21 is highly produced in Helicobacter pylori-infected gastric mucosa and promotes gelatinases synthesis. *J Immunol* **178,** 5957-5965.

Cascales, E. and Christie, P.J. (2003). The versatile bacterial type IV secretion systems. *Nature reviews. Microbiology* **1,** 137-149.

Censini, S., Lange, C., Xiang, Z., Crabtree, J.E., Ghiara, P., Borodovsky, M.*, et al.* (1996). cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* **93,** 14648-14653.

Chakraborti, S., Mandal, M., Das, S., Mandal, A. and Chakraborti, T. (2003). Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* **253,** 269-285.

Chan, A.O., Lam, S.K., Wong, B.C., Wong, W.M., Yuen, M.F., Yeung, Y.H.*, et al.* (2003a). Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer. *Gut* **52,** 502-506.

Chan, A.O., Peng, J.Z., Lam, S.K., Lai, K.C., Yuen, M.F., Cheung, H.K.*, et al.* (2006). Eradication of Helicobacter pylori infection reverses E-cadherin promoter hypermethylation. *Gut* **55,** 463-468.

Chan, A.O., Wong, B.C., Lan, H.Y., Loke, S.L., Chan, W.K., Hui, W.M.*, et al.* (2003b). Deregulation of E-cadherin-catenin complex in precancerous lesions of gastric adenocarcinoma. *J Gastroenterol Hepatol* **18,** 534-539.

Chang, J.H., Gill, S., Settleman, J. and Parsons, S.J. (1995). c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. *J Cell Biol* **130,** 355-368.

Chen, G., Sordillo, E.M., Ramey, W.G., Reidy, J., Holt, P.R., Krajewski, S.*, et al.* (1997). Apoptosis in gastric epithelial cells is induced by Helicobacter pylori and accompanied by increased expression of BAK. *Biochem Biophys Res Commun* **239,** 626-632.

Chen, J.D., Kearns, S., Porter, T., Richards, F.M., Maher, E.R. and Teh, B.T. (2001). MET mutation and familial gastric cancer. *Journal of medical genetics* **38,** E26.

Chen, S.Y. and Chen, H.C. (2006). Direct interaction of focal adhesion kinase (FAK) with Met is required for FAK to promote hepatocyte growth factor-induced cell invasion. *Mol Cell Biol* **26,** 5155-5167.

Chiang, G.J., Billmeyer, B.R., Canes, D., Stoffel, J., Moinzadeh, A., Austin, C.A.*, et al.* (2005). The src-family kinase inhibitor PP2 suppresses the in vitro invasive phenotype of bladder carcinoma cells via modulation of Akt. *BJU Int* **96,** 416-422.

Chmielowiec, J., Borowiak, M., Morkel, M., Stradal, T., Munz, B., Werner, S.*, et al.* (2007). c-Met is essential for wound healing in the skin. *J Cell Biol* **177,** 151-162.

Chung, W.C., Jung, S.H., Lee, K.M., Paik, C.N., Kawk, J.W., Jung, J.H.*, et al.* (2010). The detection of Helicobacter pylori cag pathogenicity islands (PAIs) and expression of matrix metalloproteinase-7 (MMP-7) in gastric epithelial dysplasia and intramucosal cancer. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association* **13,** 162-169.

Churin, Y., Al-Ghoul, L., Kepp, O., Meyer, T.F., Birchmeier, W. and Naumann, M. (2003). Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol* **161,** 249-255.

Churin, Y., Kardalinou, E., Meyer, T.F. and Naumann, M. (2001). Pathogenicity island-dependent activation of Rho GTPases Rac1 and Cdc42 in Helicobacter pylori infection. *Mol Microbiol* **40,** 815-823.

Conlin, V.S., Curtis, S.B., Zhao, Y., Moore, E.D., Smith, V.C., Meloche, R.M.*, et al.* (2004). Helicobacter pylori infection targets adherens junction regulatory proteins and results in increased rates of migration in human gastric epithelial cells. *Infect Immun* **72,** 5181-5192.

Correa, P. (1992). Human gastric carcinogenesis: a multistep and multifactorial process--First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* **52,** 6735-6740.

Correa, P., Fontham, E.T., Bravo, J.C., Bravo, L.E., Ruiz, B., Zarama, G.*, et al.* (2000). Chemoprevention of gastric dysplasia: randomized trial of antioxidant supplements and anti-helicobacter pylori therapy. *J Natl Cancer Inst* **92,** 1881-1888.

Cover, T.L. and Blanke, S.R. (2005). Helicobacter pylori VacA, a paradigm for toxin multifunctionality. *Nature reviews. Microbiology* **3,** 320-332.

Cover, T.L. and Blaser, M.J. (1992). Purification and characterization of the vacuolating toxin from Helicobacter pylori. *J Biol Chem* **267,** 10570-10575.

Cover, T.L., Krishna, U.S., Israel, D.A. and Peek, R.M., Jr. (2003). Induction of gastric epithelial cell apoptosis by Helicobacter pylori vacuolating cytotoxin. *Cancer Res* **63,** 951-957.

Coyle, W.J., Sedlack, R.E., Nemec, R., Peterson, R., Duntemann, T., Murphy, M. and Lawson, J.M. (1999). Eradication of Helicobacter pylori normalizes elevated mucosal levels of epidermal growth factor and its receptor. *Am J Gastroenterol* **94,** 2885-2889.

Crabtree, J.E., Shallcross, T.M., Heatley, R.V. and Wyatt, J.I. (1991a). Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut* **32,** 1473-1477.

Crabtree, J.E., Taylor, J.D., Wyatt, J.I., Heatley, R.V., Shallcross, T.M., Tompkins, D.S. and Rathbone, B.J. (1991b). Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. *Lancet* **338,** 332-335.

Crabtree, J.E., Xiang, Z., Lindley, I.J., Tompkins, D.S., Rappuoli, R. and Covacci, A. (1995). Induction of interleukin-8 secretion from gastric epithelial cells by a cagA negative isogenic mutant of Helicobacter pylori. *J Clin Pathol* **48,** 967-969.

Crawford, H.C., Krishna, U.S., Israel, D.A., Matrisian, L.M., Washington, M.K. and Peek, R.M., Jr. (2003). Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. *Gastroenterology* **125,** 1125-1136.

Crepaldi, T., Pollack, A.L., Prat, M., Zborek, A., Mostov, K. and Comoglio, P.M. (1994). Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. *J Cell Biol* **125,** 313-320.

Danese, S., Papa, A., Gasbarrini, A., Ricci, R. and Maggiano, N. (2004). Helicobacter pylori eradication down-regulates matrix metalloproteinase-9 expression in chronic gastritis and gastric ulcer. *Gastroenterology* **126,** 369-371.

Daniel, J.M. and Reynolds, A.B. (1999). The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. *Mol Cell Biol* **19,** 3614-3623.

de Martel, C., Ferlay, J., Franceschi, S., Vignat, J., Bray, F., Forman, D. and Plummer, M. (2012). Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *The lancet oncology* **13,** 607-615.

Deraz, E.M., Kudo, Y., Yoshida, M., Obayashi, M., Tsunematsu, T., Tani, H.*, et al.* (2011). MMP-10/stromelysin-2 promotes invasion of head and neck cancer. *PLoS One* **6,** e25438.

Ding, S.Z., Minohara, Y., Fan, X.J., Wang, J., Reyes, V.E., Patel, J.*, et al.* (2007). Helicobacter pylori infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infect Immun* **75,** 4030-4039.

Du, Y., Danjo, K., Robinson, P.A. and Crabtree, J.E. (2007). In-Cell Western analysis of Helicobacter pylori-induced phosphorylation of extracellular-signal related kinase via the transactivation of the epidermal growth factor receptor. *Microbes Infect* **9,** 838-846.

Eftang, L.L., Esbensen, Y., Tannaes, T.M., Bukholm, I.R. and Bukholm, G. (2012). Interleukin-8 is the single most up-regulated gene in whole genome profiling of H. pylori exposed gastric epithelial cells. *BMC Microbiol* **12,** 9.

Egeblad, M. and Werb, Z. (2002). New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* **2,** 161-174.

El-Etr, S.H., Mueller, A., Tompkins, L.S., Falkow, S. and Merrell, D.S. (2004). Phosphorylation-independent effects of CagA during interaction between Helicobacter pylori and T84 polarized monolayers. *J Infect Dis* **190,** 1516-1523.

El-Omar, E.M., Carrington, M., Chow, W.H., McColl, K.E., Bream, J.H., Young, H.A.*, et al.* (2000). Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* **404,** 398-402.

El-Omar, E.M., Rabkin, C.S., Gammon, M.D., Vaughan, T.L., Risch, H.A., Schoenberg, J.B.*, et al.* (2003). Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymorphisms. *Gastroenterology* **124,** 1193-1201.

el-Shoura, S.M. (1995). Helicobacter pylori: I. Ultrastructural sequences of adherence, attachment, and penetration into the gastric mucosa. *Ultrastructural pathology* **19,** 323-333.

Elfvin, A., Edebo, A., Bolin, I. and Fandriks, L. (2007). Quantitative measurement of nitric oxide and hydrogen peroxide in Helicobacter pylori-infected Mongolian gerbils in vivo. *Scand J Gastroenterol* **42,** 1175-1181.

Engstrand, L., Graham, D., Scheynius, A., Genta, R.M. and El-Zaatari, F. (1997). Is the sanctuary where Helicobacter pylori avoids antibacterial treatment intracellular? *American journal of clinical pathology* **108,** 504-509.

Falush, D., Wirth, T., Linz, B., Pritchard, J.K., Stephens, M., Kidd, M.*, et al.* (2003). Traces of human migrations in Helicobacter pylori populations. *Science* **299,** 1582-1585.

Fan, X.G., Kelleher, D., Fan, X.J., Xia, H.X. and Keeling, P.W. (1996). Helicobacter pylori increases proliferation of gastric epithelial cells. *Gut* **38,** 19-22.

Fedwick, J.P., Lapointe, T.K., Meddings, J.B., Sherman, P.M. and Buret, A.G. (2005). Helicobacter pylori activates myosin light-chain kinase to disrupt claudin-4 and claudin-5 and increase epithelial permeability. *Infect Immun* **73,** 7844-7852.

Ferraro, D., Corso, S., Fasano, E., Panieri, E., Santangelo, R., Borrello, S.*, et al.* (2006). Pro-metastatic signaling by c-Met through RAC-1 and reactive oxygen species (ROS). *Oncogene* **25,** 3689-3698.

Ferreira, A.C., Suriano, G., Mendes, N., Gomes, B., Wen, X., Carneiro, F.*, et al.* (2012). E-cadherin impairment increases cell survival through Notch-dependent upregulation of Bcl-2. *Hum Mol Genet* **21,** 334-343.

Figueiredo, C., Machado, J.C., Pharoah, P., Seruca, R., Sousa, S., Carvalho, R.*, et al.* (2002). Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* **94,** 1680-1687.

Franco, A.T., Israel, D.A., Washington, M.K., Krishna, U., Fox, J.G., Rogers, A.B.*, et al.* (2005). Activation of beta-catenin by carcinogenic Helicobacter pylori. *Proc Natl Acad Sci U S A* **102,** 10646-10651.

Franco, A.T., Johnston, E., Krishna, U., Yamaoka, Y., Israel, D.A., Nagy, T.A.*, et al.* (2008). Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors. *Cancer Res* **68,** 379-387.

Franke, R., Muller, M., Wundrack, N., Gilles, E.D., Klamt, S., Kahne, T. and Naumann, M. (2008). Host-pathogen systems biology: logical modelling of hepatocyte growth factor and Helicobacter pylori induced c-Met signal transduction. *BMC Syst Biol* **2,** 4.

Frixen, U.H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A.*, et al.* (1991). E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* **113,** 173-185.

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H.E., Behrens, J.*, et al.* (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* **4,** 222-231.

Furge, K.A., Zhang, Y.W. and Vande Woude, G.F. (2000). Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* **19,** 5582-5589.

Galmiche, A., Rassow, J., Doye, A., Cagnol, S., Chambard, J.C., Contamin, S.*, et al.* (2000). The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J* **19,** 6361-6370.

Garcia-Guzman, M., Larsen, E. and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* **19,** 4058-4065.

Gartner, F., David, L., Seruca, R., Machado, J.C. and Sobrinho-Simoes, M. (1996). Establishment and characterization of two cell lines derived from human diffuse gastric carcinomas xenografted in nude mice. *Virchows Archiv : an international journal of pathology* **428,** 91-98.

Ghose, C., Perez-Perez, G.I., Dominguez-Bello, M.G., Pride, D.T., Bravi, C.M. and Blaser, M.J. (2002). East Asian genotypes of Helicobacter pylori strains in Amerindians provide evidence for its ancient human carriage. *Proc Natl Acad Sci U S A* **99,** 15107-15111.

Giannakis, M., Chen, S.L., Karam, S.M., Engstrand, L. and Gordon, J.I. (2008). Helicobacter pylori evolution during progression from chronic atrophic gastritis to gastric cancer and its impact on gastric stem cells. *Proc Natl Acad Sci U S A* **105,** 4358-4363.

Gill, J.H., Kirwan, I.G., Seargent, J.M., Martin, S.W., Tijani, S., Anikin, V.A.*, et al.* (2004). MMP-10 is overexpressed, proteolytically active, and a potential target for therapeutic intervention in human lung carcinomas. *Neoplasia* **6,** 777-785.

Giordano, S., Di Renzo, M.F., Narsimhan, R.P., Cooper, C.S., Rosa, C. and Comoglio, P.M. (1989). Biosynthesis of the protein encoded by the c-met proto-oncogene. *Oncogene* **4,** 1383-1388.

Giordano, S., di Renzo, M.F., Olivero, M., Mondino, A., Zhen, Z., Medico, E. and Comoglio, P.M. (1992). The c-met/HGF receptor in human tumours. *Eur J Cancer Prev* **1 Suppl 3,** 45-49.

Gnad, T., Feoktistova, M., Leverkus, M., Lendeckel, U. and Naumann, M. (2010). Helicobacter pylori-induced activation of beta-catenin involves low density lipoprotein receptor-related protein 6 and Dishevelled. *Mol Cancer* **9,** 31.

Gooding, J.M., Yap, K.L. and Ikura, M. (2004). The cadherin-catenin complex as a focal point of cell adhesion and signalling: new insights from three-dimensional structures. *Bioessays* **26,** 497-511.

Gooz, M., Gooz, P. and Smolka, A.J. (2001). Epithelial and bacterial metalloproteinases and their inhibitors in H. pylori infection of human gastric cells. *Am J Physiol Gastrointest Liver Physiol* **281,** G823-832.

Gooz, M., Shaker, M., Gooz, P. and Smolka, A.J. (2003). Interleukin 1beta induces gastric epithelial cell matrix metalloproteinase secretion and activation during Helicobacter pylori infection. *Gut* **52,** 1250-1256.

Gruenheid, S., DeVinney, R., Bladt, F., Goosney, D., Gelkop, S., Gish, G.D.*, et al.* (2001). Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. *Nat Cell Biol* **3,** 856-859.

Guillemin, K., Salama, N.R., Tompkins, L.S. and Falkow, S. (2002). Cag pathogenicity island-specific responses of gastric epithelial cells to Helicobacter pylori infection. *Proc Natl Acad Sci U S A* **99,** 15136-15141.

Gusterson, B., Cowley, G., Smith, J.A. and Ozanne, B. (1984). Cellular localisation of human epidermal growth factor receptor. *Cell biology international reports* **8,** 649-658.

Hake, M.J., Choowongkomon, K., Kostenko, O., Carlin, C.R. and Sonnichsen, F.D. (2008). Specificity determinants of a novel Nck interaction with the juxtamembrane domain of the epidermal growth factor receptor. *Biochemistry* **47,** 3096-3108.

Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. (1993). p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J* **12,** 307-314.

Hansson, L.E., Nyren, O., Bergstrom, R., Wolk, A., Lindgren, A., Baron, J. and Adami, H.O. (1993). Diet and risk of gastric cancer. A population-based case-control study in Sweden. *Int J Cancer* **55,** 181-189.

Hartsock, A. and Nelson, W.J. (2008). Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta* **1778,** 660-669.

Hasegawa, Y., Yamamoto, M., Maeda, S. and Saitoh, Y. (1995). Hepatocyte growth-factor and its receptor C-met regulate both cell-growth and invasion of human pancreatic-cancer. *Int J Oncol* **7,** 877-881.

Hatakeyama, M. (2004). Oncogenic mechanisms of the Helicobacter pylori CagA protein. *Nat Rev Cancer* **4,** 688-694.

Hazell, S.L., Lee, A., Brady, L. and Hennessy, W. (1986). Campylobacter pyloridis and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. *J Infect Dis* **153,** 658-663.

Herynk, M.H., Zhang, J., Parikh, N.U. and Gallick, G.E. (2007). Activation of Src by c-Met overexpression mediates metastatic properties of colorectal carcinoma cells. *J Exp Ther Oncol* **6,** 205-217.

Higashi, H., Tsutsumi, R., Muto, S., Sugiyama, T., Azuma, T., Asaka, M. and Hatakeyama, M. (2002). SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein. *Science* **295,** 683-686.

Higashi, H., Yokoyama, K., Fujii, Y., Ren, S., Yuasa, H., Saadat, I.*, et al.* (2005). EPIYA motif is a membrane-targeting signal of Helicobacter pylori virulence factor CagA in mammalian cells. *J Biol Chem* **280,** 23130-23137.

Hirao, T., Sawada, H., Koyama, F., Watanabe, A., Yamada, Y., Sakaguchi, T.*, et al.* (1999). Antisense epidermal growth factor receptor delivered by adenoviral vector blocks tumor growth in human gastric cancer. *Cancer gene therapy* **6,** 423-427.

Hiscox, S. and Jiang, W.G. (1999). Association of the HGF/SF receptor, c-met, with the cell-surface adhesion molecule, E-cadherin, and catenins in human tumor cells. *Biochem Biophys Res Commun* **261,** 406-411.

Hori, K., Shiota, G. and Kawasaki, H. (2000). Expression of hepatocyte growth factor and c-met receptor in gastric mucosa during gastric ulcer healing. *Scand J Gastroenterol* **35,** 23-31.

Hoschuetzky, H., Aberle, H. and Kemler, R. (1994). Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J Cell Biol* **127,** 1375-1380.

Hoy, B., Geppert, T., Boehm, M., Reisen, F., Plattner, P., Gadermaier, G.*, et al.* (2012). Distinct roles of secreted HtrA proteases from gram-negative pathogens in cleaving the junctional protein and tumor suppressor E-cadherin. *J Biol Chem* **287,** 10115-10120.

Hoy, B., Lower, M., Weydig, C., Carra, G., Tegtmeyer, N., Geppert, T.*, et al.* (2010). Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion. *EMBO Rep* **11,** 798-804.

Hsi, L.C., Angerman-Stewart, J. and Eling, T.E. (1999). Introduction of full-length APC modulates cyclooxygenase-2 expression in HT-29 human colorectal carcinoma cells at the translational level. *Carcinogenesis* **20,** 2045-2049.

Huang, F.Y., Chan, A.O., Rashid, A., Wong, D.K., Cho, C.H. and Yuen, M.F. (2012). Helicobacter pylori induces promoter methylation of E-cadherin via interleukin-1beta activation of nitric oxide production in gastric cancer cells. *Cancer*.

Hubbard, S.R. and Till, J.H. (2000). Protein tyrosine kinase structure and function. *Annual review of biochemistry* **69,** 373-398.

Huh, C.G., Factor, V.M., Sanchez, A., Uchida, K., Conner, E.A. and Thorgeirsson, S.S. (2004). Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A* **101,** 4477-4482.

Hui, A.Y., Meens, J.A., Schick, C., Organ, S.L., Qiao, H., Tremblay, E.A.*, et al.* (2009). Src and FAK mediate cell-matrix adhesion-dependent activation of Met during transformation of breast epithelial cells. *J Cell Biochem* **107,** 1168-1181.

Hung, K.H., Wu, J.J., Yang, H.B., Su, L.J. and Sheu, B.S. (2009). Host Wnt/beta-catenin pathway triggered by Helicobacter pylori correlates with regression of gastric intestinal metaplasia after H. pylori eradication. *J Med Microbiol* **58,** 567-576.

Inoshima, I., Inoshima, N., Wilke, G.A., Powers, M.E., Frank, K.M., Wang, Y. and Bubeck Wardenburg, J. (2011). A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. *Nat Med* **17,** 1310-1314.

Inoue, T., Kataoka, H., Goto, K., Nagaike, K., Igami, K., Naka, D.*, et al.* (2004). Activation of c-Met (hepatocyte growth factor receptor) in human gastric cancer tissue. *Cancer Sci* **95,** 803-808.

Iwamoto, H., Czajkowsky, D.M., Cover, T.L., Szabo, G. and Shao, Z. (1999). VacA from Helicobacter pylori: a hexameric chloride channel. *FEBS Lett* **450,** 101-104.

Jagadeeswaran, R., Jagadeeswaran, S., Bindokas, V.P. and Salgia, R. (2007). Activation of HGF/c-Met pathway contributes to the reactive oxygen species generation and motility of small cell lung cancer cells. *Am J Physiol Lung Cell Mol Physiol* **292,** L1488-1494.

Jamora, C. and Fuchs, E. (2002). Intercellular adhesion, signalling and the cytoskeleton. *Nat Cell Biol* **4,** E101-108.

Jiang, W.G., Martin, T.A., Parr, C., Davies, G., Matsumoto, K. and Nakamura, T. (2005). Hepatocyte growth factor, its receptor, and their potential value in cancer therapies. *Critical reviews in oncology/hematology* **53,** 35-69.

Jo, M., Stolz, D.B., Esplen, J.E., Dorko, K., Michalopoulos, G.K. and Strom, S.C. (2000). Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. *J Biol Chem* **275,** 8806-8811.

Joh, T., Kataoka, H., Tanida, S., Watanabe, K., Ohshima, T., Sasaki, M.*, et al.* (2005). Helicobacter pylori-stimulated interleukin-8 (IL-8) promotes cell proliferation through transactivation of epidermal growth factor receptor (EGFR) by disintegrin and metalloproteinase (ADAM) activation. *Dig Dis Sci* **50,** 2081-2089.

Johnson, L.G. (2005). Applications of imaging techniques to studies of epithelial tight junctions. *Advanced drug delivery reviews* **57,** 111-121.

Jones, N.P., Peak, J., Brader, S., Eccles, S.A. and Katan, M. (2005). PLCgamma1 is essential for early events in integrin signalling required for cell motility. *J Cell Sci* **118,** 2695-2706.

Kaji, M., Yonemura, Y., Harada, S., Liu, X., Terada, I. and Yamamoto, H. (1996). Participation of c-met in the progression of human gastric cancers: anti-c-met oligonucleotides inhibit proliferation or invasiveness of gastric cancer cells. *Cancer gene therapy* **3,** 393-404.

Kassis, J., Moellinger, J., Lo, H., Greenberg, N.M., Kim, H.G. and Wells, A. (1999). A role for phospholipase C-gamma-mediated signaling in tumor cell invasion. *Clin Cancer Res* **5,** 2251-2260.

Katsurahara, M., Kobayashi, Y., Iwasa, M., Ma, N., Inoue, H., Fujita, N.*, et al.* (2009). Reactive nitrogen species mediate DNA damage in Helicobacter pylori-infected gastric mucosa. *Helicobacter* **14,** 552-558.

Katz, J., Sambandam, V., Wu, J.H., Michalek, S.M. and Balkovetz, D.F. (2000). Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. *Infect Immun* **68,** 1441-1449.

Kaurah, P., MacMillan, A., Boyd, N., Senz, J., De Luca, A., Chun, N.*, et al.* (2007). Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. *JAMA* **297,** 2360-2372.

Keates, S., Keates, A.C., Katchar, K., Peek, R.M., Jr. and Kelly, C.P. (2007). Helicobacter pylori induces up-regulation of the epidermal growth factor receptor in AGS gastric epithelial cells. *J Infect Dis* **196,** 95-103.

Keates, S., Keates, A.C., Nath, S., Peek, R.M., Jr. and Kelly, C.P. (2005). Transactivation of the epidermal growth factor receptor by cag+ Helicobacter pylori induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells. *Gut* **54,** 1363-1369.

Keates, S., Sougioultzis, S., Keates, A.C., Zhao, D., Peek, R.M., Jr., Shaw, L.M. and Kelly, C.P. (2001). cag+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. *J Biol Chem* **276,** 48127-48134.

Kidd, M., Lastovica, A.J., Atherton, J.C. and Louw, J.A. (1999). Heterogeneity in the Helicobacter pylori vacA and cagA genes: association with gastroduodenal disease in South Africa? *Gut* **45,** 499-502.

Kim, J.J., Tao, H., Carloni, E., Leung, W.K., Graham, D.Y. and Sepulveda, A.R. (2002a). Helicobacter pylori impairs DNA mismatch repair in gastric epithelial cells. *Gastroenterology* **123,** 542-553.

Kim, J.M., Kim, J.S., Jung, H.C., Song, I.S. and Kim, C.Y. (2002b). Up-regulation of inducible nitric oxide synthase and nitric oxide in Helicobacter pylori-infected human gastric epithelial cells: possible role of interferon-gamma in polarized nitric oxide secretion. *Helicobacter* **7,** 116-128.

Kim, L. and Wong, T.W. (1995). The cytoplasmic tyrosine kinase FER is associated with the catenin-like substrate pp120 and is activated by growth factors. *Mol Cell Biol* **15,** 4553-4561.

Kirikoshi, H., Sekihara, H. and Katoh, M. (2001). Up-regulation of WNT10A by tumor necrosis factor alpha and Helicobacter pylori in gastric cancer. *Int J Oncol* **19,** 533-536.

Kitadai, Y., Sasaki, A., Ito, M., Tanaka, S., Oue, N., Yasui, W.*, et al.* (2003). Helicobacter pylori infection influences expression of genes related to angiogenesis and invasion in human gastric carcinoma cells. *Biochem Biophys Res Commun* **311,** 809-814.

Kochhar, K.S. and Iyer, A.P. (1996). Hepatocyte growth factor induces activation of Nck and phospholipase C-gamma in lung carcinoma cells. *Cancer Lett* **104,** 163-169.

Kodama, A., Matozaki, T., Fukuhara, A., Kikyo, M., Ichihashi, M. and Takai, Y. (2000). Involvement of an SHP-2-Rho small G protein pathway in hepatocyte growth factor/scatter factor-induced cell scattering. *Mol Biol Cell* **11,** 2565-2575.

Konturek, P.K., Brzozowski, T., Konturek, S.J. and Dembinski, A. (1990). Role of epidermal growth factor, prostaglandin, and sulfhydryls in stress-induced gastric lesions. *Gastroenterology* **99,** 1607-1615.

Koyama, S. (2004). Significance of cell-surface expression of matrix metalloproteinases and their inhibitors on gastric epithelium and infiltrating mucosal lymphocytes in progression of Helicobacter pylori-associated gastritis. *Scand J Gastroenterol* **39,** 1046-1053.

Krueger, S., Hundertmark, T., Kalinski, T., Peitz, U., Wex, T., Malfertheiner, P.*, et al.* (2006). Helicobacter pylori encoding the pathogenicity island activates matrix metalloproteinase 1 in gastric epithelial cells via JNK and ERK. *J Biol Chem* **281,** 2868-2875.

Krueger, S., Hundertmark, T., Kuester, D., Kalinski, T., Peitz, U. and Roessner, A. (2007). Helicobacter pylori alters the distribution of ZO-1 and p120ctn in primary human gastric epithelial cells. *Pathol Res Pract* **203,** 433-444.

Kubben, F.J., Sier, C.F., Schram, M.T., Witte, A.M., Veenendaal, R.A., van Duijn, W.*, et al.* (2007). Eradication of Helicobacter pylori infection favourably affects altered gastric mucosal MMP-9 levels. *Helicobacter* **12,** 498-504.

Kubicka, S., Claas, C., Staab, S., Kuhnel, F., Zender, L., Trautwein, C.*, et al.* (2002). p53 mutation pattern and expression of c-erbB2 and c-met in gastric cancer: relation to histological subtypes, Helicobacter pylori infection, and prognosis. *Dig Dis Sci* **47,** 114-121.

Kuck, D., Kolmerer, B., Iking-Konert, C., Krammer, P.H., Stremmel, W. and Rudi, J. (2001). Vacuolating cytotoxin of Helicobacter pylori induces apoptosis in the human gastric epithelial cell line AGS. *Infect Immun* **69,** 5080-5087.

Kuipers, E.J., Israel, D.A., Kusters, J.G., Gerrits, M.M., Weel, J., van Der Ende, A.*, et al.* (2000). Quasispecies development of Helicobacter pylori observed in paired isolates obtained years apart from the same host. *J Infect Dis* **181,** 273-282.

Kundu, P., De, R., Pal, I., Mukhopadhyay, A.K., Saha, D.R. and Swarnakar, S. (2011). Curcumin alleviates matrix metalloproteinase-3 and -9 activities during eradication of Helicobacter pylori infection in cultured cells and mice. *PLoS One* **6,** e16306.

Kundu, P., Mukhopadhyay, A.K., Patra, R., Banerjee, A., Berg, D.E. and Swarnakar, S. (2006). Cag pathogenicity island-independent up-regulation of matrix metalloproteinases-9 and -2 secretion and expression in mice by Helicobacter pylori infection. *J Biol Chem* **281,** 34651-34662.

Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H. and Tahara, E. (1992). Frequent amplification of the c-met gene in scirrhous type stomach cancer. *Biochem Biophys Res Commun* **189,** 227-232.

Kurashima, Y., Murata-Kamiya, N., Kikuchi, K., Higashi, H., Azuma, T., Kondo, S. and Hatakeyama, M. (2008). Deregulation of beta-catenin signal by Helicobacter pylori CagA requires the CagA-multimerization sequence. *Int J Cancer* **122,** 823-831.

Kusters, J.G., van Vliet, A.H. and Kuipers, E.J. (2006). Pathogenesis of Helicobacter pylori infection. *Clin Microbiol Rev* **19,** 449-490.

Laird, D.W. (2006). Life cycle of connexins in health and disease. *Biochem J* **394,** 527-543.

Lapointe, T.K., O'Connor, P.M., Jones, N.L., Menard, D. and Buret, A.G. (2010). Interleukin-1 receptor phosphorylation activates Rho kinase to disrupt human gastric tight junctional claudin-4 during Helicobacter pylori infection. *Cell Microbiol* **12,** 692-703.

Lawler, K., O'Sullivan, G., Long, A. and Kenny, D. (2009). Shear stress induces internalization of E-cadherin and invasiveness in metastatic oesophageal cancer cells by a Src-dependent pathway. *Cancer Sci* **100,** 1082-1087.

Lecuit, M., Hurme, R., Pizarro-Cerda, J., Ohayon, H., Geiger, B. and Cossart, P. (2000). A role for alpha-and beta-catenins in bacterial uptake. *Proc Natl Acad Sci U S A* **97,** 10008-10013.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C.*, et al.* (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science* **292,** 1722-1725.

Lee, J., Kang, W.K., Park, J.O., Park, S.H., Park, Y.S., Lim, H.Y.*, et al.* (2009). Expression of activated signal transducer and activator of transcription 3 predicts poor clinical outcome in gastric adenocarcinoma. *APMIS* **117,** 598-606.

Lemmon, M.A. and Schlessinger, J. (1994). Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends in biochemical sciences* **19,** 459-463.

Lemmon, M.A. and Schlessinger, J. (1998). Transmembrane signaling by receptor oligomerization. *Methods Mol Biol* **84,** 49-71.

Leung, W.K., Man, E.P., Yu, J., Go, M.Y., To, K.F., Yamaoka, Y.*, et al.* (2006). Effects of Helicobacter pylori eradication on methylation status of E-cadherin gene in noncancerous stomach. *Clin Cancer Res* **12,** 3216-3221.

Lin, M.T., Lin, B.R., Chang, C.C., Chu, C.Y., Su, H.J., Chen, S.T.*, et al.* (2007). IL-6 induces AGS gastric cancer cell invasion via activation of the c-Src/RhoA/ROCK signaling pathway. *Int J Cancer* **120,** 2600-2608.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W.*, et al.* (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350,** 2129-2139.

Machado, A.M., Figueiredo, C., Touati, E., Maximo, V., Sousa, S., Michel, V.*, et al.* (2009). Helicobacter pylori infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells. *Clin Cancer Res* **15,** 2995-3002.

Machado, J.C., Figueiredo, C., Canedo, P., Pharoah, P., Carvalho, R., Nabais, S.*, et al.* (2003). A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. *Gastroenterology* **125,** 364-371.

Machado, J.C., Oliveira, C., Carvalho, R., Soares, P., Berx, G., Caldas, C.*, et al.* (2001a). E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. *Oncogene* **20,** 1525-1528.

Machado, J.C., Pharoah, P., Sousa, S., Carvalho, R., Oliveira, C., Figueiredo, C.*, et al.* (2001b). Interleukin 1B and interleukin 1RN polymorphisms are associated with increased risk of gastric carcinoma. *Gastroenterology* **121,** 823-829.

Maeda, S., Yoshida, H., Mitsuno, Y., Hirata, Y., Ogura, K., Shiratori, Y. and Omata, M. (2002). Analysis of apoptotic and antiapoptotic signalling pathways induced by Helicobacter pylori. *Molecular pathology : MP* **55,** 286-293.

Mannello, F. and Medda, V. Nuclear localization of matrix metalloproteinases. *Prog Histochem Cytochem* **47,** 27-58.

Maratos-Flier, E., Kao, C.Y., Verdin, E.M. and King, G.L. (1987). Receptor-mediated vectorial transcytosis of epidermal growth factor by Madin-Darby canine kidney cells. *J Cell Biol* **105,** 1595-1601.

Marchenko, G.N., Marchenko, N.D., Leng, J. and Strongin, A.Y. (2002). Promoter characterization of the novel human matrix metalloproteinase-26 gene: regulation by the T-cell factor-4 implies specific expression of the gene in cancer cells of epithelial origin. *Biochem J* **363,** 253-262.

Mareel, M. and Leroy, A. (2003). Clinical, cellular, and molecular aspects of cancer invasion. *Physiological reviews* **83,** 337-376.

Marshall, B.J. and Warren, J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **1,** 1311-1315.

Mateus, A.R., Seruca, R., Machado, J.C., Keller, G., Oliveira, M.J., Suriano, G. and Luber, B. (2007). EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells. *Hum Mol Genet* **16,** 1639-1647.

Mathew, R., Khanna, R., Kumar, R., Mathur, M., Shukla, N.K. and Ralhan, R. (2002). Stromelysin-2 overexpression in human esophageal squamous cell carcinoma: potential clinical implications. *Cancer Detect Prev* **26,** 222-228.

Matsuuchi, L. and Naus, C.C. (2012). Gap Junction Proteins on the Move: Connexins, the Cytoskeleton and Migration. *Biochim Biophys Acta*.

Matter, K. and Balda, M.S. (2003). Functional analysis of tight junctions. *Methods* **30,** 228-234.

McCaig, C., Duval, C., Hemers, E., Steele, I., Pritchard, D.M., Przemeck, S.*, et al.* (2006). The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to Helicobacter pylori. *Gastroenterology* **130,** 1754-1763.

McCawley, L.J. and Matrisian, L.M. (2001). Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* **13,** 534-540.

McLachlan, R.W., Kraemer, A., Helwani, F.M., Kovacs, E.M. and Yap, A.S. (2007). E-cadherin adhesion activates c-Src signaling at cell-cell contacts. *Mol Biol Cell* **18,** 3214-3223.

Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M. and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. *Cell* **84,** 923-932.

Miehlke, S., Kirsch, C., Agha-Amiri, K., Gunther, T., Lehn, N., Malfertheiner, P.*, et al.* (2000). The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. *Int J Cancer* **87,** 322-327.

Mimuro, H., Suzuki, T., Tanaka, J., Asahi, M., Haas, R. and Sasakawa, C. (2002). Grb2 is a key mediator of helicobacter pylori CagA protein activities. *Molecular cell* **10,** 745-755.

Moens, E. and Veldhoen, M. (2012). Epithelial barrier biology: good fences make good neighbours. *Immunology* **135,** 1-8.

Monteagudo, C., Merino, M.J., San-Juan, J., Liotta, L.A. and Stetler-Stevenson, W.G. (1990). Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. *Am J Pathol* **136,** 585-592.

Montecucco, C. and Rappuoli, R. (2001). Living dangerously: how Helicobacter pylori survives in the human stomach. *Nat Rev Mol Cell Biol* **2,** 457-466.

Mori, N., Sato, H., Hayashibara, T., Senba, M., Geleziunas, R., Wada, A.*, et al.* (2003). Helicobacter pylori induces matrix metalloproteinase-9 through activation of nuclear factor kappaB. *Gastroenterology* **124,** 983-992.

Moscatello, D.K., Holgado-Madruga, M., Godwin, A.K., Ramirez, G., Gunn, G., Zoltick, P.W.*, et al.* (1995). Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* **55,** 5536-5539.

Moss, S.F., Calam, J., Agarwal, B., Wang, S. and Holt, P.R. (1996). Induction of gastric epithelial apoptosis by Helicobacter pylori. *Gut* **38,** 498-501.

Mueller, K.L., Hunter, L.A., Ethier, S.P. and Boerner, J.L. (2008). Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. *Cancer Res* **68,** 3314-3322.

Murata-Kamiya, N., Kurashima, Y., Teishikata, Y., Yamahashi, Y., Saito, Y., Higashi, H.*, et al.* (2007). Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* **26,** 4617-4626.

Nagy, T.A., Wroblewski, L.E., Wang, D., Piazuelo, M.B., Delgado, A., Romero-Gallo, J.*, et al.* (2011). beta-Catenin and p120 Mediate PPARdelta-Dependent Proliferation Induced by Helicobacter pylori in Human and Rodent Epithelia. *Gastroenterology* **141,** 553-564.

Nakamura, H., Fujii, Y., Ohuchi, E., Yamamoto, E. and Okada, Y. (1998). Activation of the precursor of human stromelysin 2 and its interactions with other matrix metalloproteinases. *Eur J Biochem* **253,** 67-75.

Nakayama, M., Hisatsune, J., Yamasaki, E., Isomoto, H., Kurazono, H., Hatakeyama, M.*, et al.* (2009). Helicobacter pylori VacA-induced inhibition of GSK3 through the PI3K/Akt signaling pathway. *J Biol Chem* **284,** 1612-1619.

Nam, Y.H., Ryu, E., Lee, D., Shim, H.J., Lee, Y.C. and Lee, S.T. (2011). CagA phosphorylation-dependent MMP-9 expression in gastric epithelial cells. *Helicobacter* **16,** 276-283.

Nath, D., Williamson, N.J., Jarvis, R. and Murphy, G. (2001). Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. *J Cell Sci* **114,** 1213-1220.

Naumann, M. and Crabtree, J.E. (2004). Helicobacter pylori-induced epithelial cell signalling in gastric carcinogenesis. *Trends Microbiol* **12,** 29-36.

Naumann, M., Wessler, S., Bartsch, C., Wieland, B., Covacci, A., Haas, R. and Meyer, T.F. (1999). Activation of activator protein 1 and stress response kinases in epithelial cells colonized by Helicobacter pylori encoding the cag pathogenicity island. *J Biol Chem* **274,** 31655-31662.

Necchi, V., Candusso, M.E., Tava, F., Luinetti, O., Ventura, U., Fiocca, R.*, et al.* (2007). Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. *Gastroenterology* **132,** 1009-1023.

Noach, L.A., Rolf, T.M. and Tytgat, G.N. (1994). Electron microscopic study of association between Helicobacter pylori and gastric and duodenal mucosa. *J Clin Pathol* **47,** 699-704.

Nobes, C.D. and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J Cell Biol* **144,** 1235-1244.

Noe, V., Fingleton, B., Jacobs, K., Crawford, H.C., Vermeulen, S., Steelant, W.*, et al.* (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* **114,** 111-118.

Noe, V., Willems, J., Vandekerckhove, J., Roy, F.V., Bruyneel, E. and Mareel, M. (1999). Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides. *J Cell Sci* **112 (Pt 1),** 127-135.

Nogueira, C., Figueiredo, C., Carneiro, F., Gomes, A.T., Barreira, R., Figueira, P.*, et al.* (2001). Helicobacter pylori genotypes may determine gastric histopathology. *Am J Pathol* **158,** 647-654.

Nomura, A., Stemmermann, G.N., Chyou, P.H., Kato, I., Perez-Perez, G.I. and Blaser, M.J. (1991). Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* **325,** 1132-1136.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R.*, et al.* (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* **366,** 2-16.

O'Connor, P.M., Lapointe, T.K., Jackson, S., Beck, P.L., Jones, N.L. and Buret, A.G. (2010). Helicobacter Pylori Activates Calpain Via Toll-Like Receptor 2 to Disrupt Adherens Junctions in Human Gastric Epithelial Cells. *Infect Immun*.

Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W. and Haas, R. (2000). Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. *Science* **287,** 1497-1500.

Odland, G.F. (1958). The fine structure of the interrelationship of cells in the human epidermis. *The Journal of biophysical and biochemical cytology* **4,** 529-538.

Ogata, T., Shibamura, H., Tromp, G., Sinha, M., Goddard, K.A., Sakalihasan, N.*, et al.* (2005). Genetic analysis of polymorphisms in biologically relevant candidate genes in patients with abdominal aortic aneurysms. *J Vasc Surg* **41,** 1036-1042.

Ogden, S.R., Wroblewski, L.E., Weydig, C., Romero-Gallo, J., O'Brien, D.P., Israel, D.A.*, et al.* (2008). p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. *Mol Biol Cell* **19,** 4110-4121.

Okano, Y., Mizuno, K., Osada, S., Nakamura, T. and Nozawa, Y. (1993). Tyrosine phosphorylation of phospholipase C gamma in c-met/HGF receptor-stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. *Biochem Biophys Res Commun* **190,** 842-848.

Oliveira, M.J., Costa, A.C., Costa, A.M., Henriques, L., Suriano, G., Atherton, J.C.*, et al.* (2006). Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. *J Biol Chem* **281,** 34888-34896.

Oliveira, M.J., Costa, A.M., Costa, A.C., Ferreira, R.M., Sampaio, P., Machado, J.C.*, et al.* (2009). CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses Helicobacter pylori-induced cell-invasive phenotype. *J Infect Dis* **200,** 745-755.

Oser, M., Dovas, A., Cox, D. and Condeelis, J. Nck1 and Grb2 localization patterns can distinguish invadopodia from podosomes. *European journal of cell biology* **90,** 181-188.

Ozawa, M. and Ohkubo, T. (2001). Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. *J Cell Sci* **114,** 503-512.

P, O.C., Rhys-Evans, P.H. and Eccles, S.A. (2001). Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* **127,** 813-820.

Pai, R., Cover, T.L. and Tarnawski, A.S. (1999). Helicobacter pylori vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. *Biochem Biophys Res Commun* **262,** 245-250.

Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J.L., Rappuoli, R. and Montecucco, C. (1998). Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. *J Clin Invest* **102,** 813-820.

Parks, W.C., Wilson, C.L. and Lopez-Boado, Y.S. (2004). Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* **4,** 617-629.

Pece, S. and Gutkind, J.S. (2000). Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. *J Biol Chem* **275,** 41227-41233.

Peek, R.M., Jr. and Blaser, M.J. (2002). Helicobacter pylori and gastrointestinal tract adenocarcinomas. *Nat Rev Cancer* **2,** 28-37.

Peek, R.M., Jr., Miller, G.G., Tham, K.T., Perez-Perez, G.I., Zhao, X., Atherton, J.C. and Blaser, M.J. (1995). Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. *Lab Invest* **73,** 760-770.

Pelicci, G., Giordano, S., Zhen, Z., Salcini, A.E., Lanfrancone, L., Bardelli, A.*, et al.* (1995). The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene* **10,** 1631-1638.

Pelicic, V., Reyrat, J.M., Sartori, L., Pagliaccia, C., Rappuoli, R., Telford, J.L.*, et al.* (1999). Helicobacter pylori VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. *Microbiology* **145 (Pt 8),** 2043-2050.

Perez-Perez, G.I., Salomaa, A., Kosunen, T.U., Daverman, B., Rautelin, H., Aromaa, A.*, et al.* (2002). Evidence that cagA(+) Helicobacter pylori strains are disappearing more rapidly than cagA(-) strains. *Gut* **50,** 295-298.

Perri, F., Cotugno, R., Piepoli, A., Merla, A., Quitadamo, M., Gentile, A.*, et al.* (2007). Aberrant DNA methylation in non-neoplastic gastric mucosa of H. Pylori infected patients and effect of eradication. *Am J Gastroenterol* **102,** 1361-1371.

Peruzzi, B. and Bottaro, D.P. (2006). Targeting the c-Met signaling pathway in cancer. *Clin Cancer Res* **12,** 3657-3660.

Petersen, A.M., Fussing, V., Colding, H., Blom, J., Norgaard, A., Andersen, L.P. and Krogfelt, K.A. (2000). Phenotypic and genotypic characterization of Helicobacter pylori from patients with and without peptic ulcer disease. *Scand J Gastroenterol* **35,** 359-367.

Phan, Q.T., Myers, C.L., Fu, Y., Sheppard, D.C., Yeaman, M.R., Welch, W.H.*, et al.* (2007). Als3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* **5,** e64.

Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M. and de Herreros, A.G. (2001). Regulation of beta-catenin structure and activity by tyrosine phosphorylation. *J Biol Chem* **276,** 20436-20443.

Pignatelli, M. and Vessey, C.J. (1994). Adhesion molecules: novel molecular tools in tumor pathology. *Hum Pathol* **25,** 849-856.

Pillinger, M.H., Marjanovic, N., Kim, S.Y., Lee, Y.C., Scher, J.U., Roper, J.*, et al.* (2007). Helicobacter pylori stimulates gastric epithelial cell MMP-1 secretion via CagA-dependent and -independent ERK activation. *J Biol Chem* **282,** 18722-18731.

Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev* **14,** 1837-1851.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402,** 884-888.

Pyke, C., Ralfkiaer, E., Huhtala, P., Hurskainen, T., Dano, K. and Tryggvason, K. (1992). Localization of messenger RNA for Mr 72,000 and 92,000 type IV collagenases in human skin cancers by in situ hybridization. *Cancer Res* **52,** 1336-1341.

Qian, X., Huang, C., Cho, C.H., Hui, W.M., Rashid, A. and Chan, A.O. (2008). E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. *Cancer Lett* **263,** 107-113.

Raff, M.C. (1992). Social controls on cell survival and cell death. *Nature* **356,** 397-400.

Rahimi, N., Hung, W., Tremblay, E., Saulnier, R. and Elliott, B. (1998). c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *J Biol Chem* **273,** 33714-33721.

Reshetnikova, G., Troyanovsky, S. and Rimm, D.L. (2007). Definition of a direct extracellular interaction between Met and E-cadherin. *Cell Biol Int* **31,** 366-373.

Reunanen, N., Westermarck, J., Hakkinen, L., Holmstrom, T.H., Elo, I., Eriksson, J.E. and Kahari, V.M. (1998). Enhancement of fibroblast collagenase (matrix metalloproteinase-1) gene expression by ceramide is mediated by extracellular signal-regulated and stress-activated protein kinase pathways. *J Biol Chem* **273,** 5137-5145.

Reynolds, A.B. and Carnahan, R.H. (2004a). Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin Cell Dev Biol* **15,** 657-663.

Reynolds, A.B., Kanner, S.B., Wang, H.C. and Parsons, J.T. (1989). Stable association of activated pp60src with two tyrosine-phosphorylated cellular proteins. *Mol Cell Biol* **9,** 3951-3958.

Reynolds, A.B. and Roczniak-Ferguson, A. (2004b). Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* **23,** 7947-7956.

Rhead, J.L., Letley, D.P., Mohammadi, M., Hussein, N., Mohagheghi, M.A., Eshagh Hosseini, M. and Atherton, J.C. (2007). A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* **133,** 926-936.

Ridley, S.H., Sarsfield, S.J., Lee, J.C., Bigg, H.F., Cawston, T.E., Taylor, D.J.*, et al.* (1997). Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. *J Immunol* **158,** 3165-3173.

Rieke, C., Kahne, T., Schweitzer, K., Schraven, B., Wienands, J., Engelke, M. and Naumann, M. (2010). Non-T cell activation linker regulates ERK activation in Helicobacter pylori-infected epithelial cells. *Cell Signal* **22,** 395-403.

Rivera, G.M., Antoku, S., Gelkop, S., Shin, N.Y., Hanks, S.K., Pawson, T. and Mayer, B.J. (2006). Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. *Proc Natl Acad Sci U S A* **103,** 9536-9541.

Roczniak-Ferguson, A. and Reynolds, A.B. (2003). Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci* **116,** 4201-4212.

Romero-Adrian, T.B., Leal-Montiel, J., Monsalve-Castillo, F., Mengual-Moreno, E., McGregor, E.G., Perini, L. and Antunez, A. (2010). Helicobacter pylori: bacterial factors and the role of cytokines in the immune response. *Current microbiology* **60,** 143-155.

Rothenbacher, D. and Brenner, H. (2003). Burden of Helicobacter pylori and H. pylori-related diseases in developed countries: recent developments and future implications. *Microbes Infect* **5,** 693-703.

Ruusala, A., Pawson, T., Heldin, C.H. and Aspenstrom, P. (2008). Nck adapters are involved in the formation of dorsal ruffles, cell migration, and Rho signaling downstream of the platelet-derived growth factor beta receptor. *J Biol Chem* **283,** 30034-30044.

Ryniers, F., Stove, C., Goethals, M., Brackenier, L., Noe, V., Bracke, M.*, et al.* (2002). Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion. *Biological chemistry* **383,** 159-165.

Saadat, I., Higashi, H., Obuse, C., Umeda, M., Murata-Kamiya, N., Saito, Y.*, et al.* (2007). Helicobacter pylori CagA targets PAR1/MARK kinase to disrupt epithelial cell polarity. *Nature* **447,** 330-333.

Saghizadeh, M., Brown, D.J., Castellon, R., Chwa, M., Huang, G.H., Ljubimova, J.Y.*, et al.* (2001). Overexpression of matrix metalloproteinase-10 and matrix metalloproteinase-3 in human diabetic corneas: a possible mechanism of basement membrane and integrin alterations. *Am J Pathol* **158,** 723-734.

Schirrmeister, W., Gnad, T., Wex, T., Higashiyama, S., Wolke, C., Naumann, M. and Lendeckel, U. (2009). Ectodomain shedding of E-cadherin and c-Met is induced by Helicobacter pylori infection. *Exp Cell Res* **315,** 3500-3508.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. *Cell* **103,** 211-225.

Segal, E.D., Cha, J., Lo, J., Falkow, S. and Tompkins, L.S. (1999). Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. *Proc Natl Acad Sci U S A* **96,** 14559-14564.

Selbach, M., Moese, S., Hauck, C.R., Meyer, T.F. and Backert, S. (2002). Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. *J Biol Chem* **277,** 6775-6778.

Shaw, G.P., Hatt, J.F., Anderson, N.G. and Hanson, P.J. (1987). Action of epidermal growth factor on acid secretion by rat isolated parietal cells. *Biochem J* **244,** 699-704.

Sheetz, M.P., Felsenfeld, D., Galbraith, C.G. and Choquet, D. (1999). Cell migration as a five-step cycle. *Biochemical Society symposium* **65,** 233-243.

Shun, C.T., Wu, M.S., Lin, M.T., Chang, M.C., Lin, J.T. and Chuang, S.M. (2001). Immunohistochemical evaluation of cadherin and catenin expression in early gastric carcinomas: correlation with clinicopathologic characteristics and Helicobacter pylori infection. *Oncology* **60,** 339-345.

Smoot, D.T., Elliott, T.B., Verspaget, H.W., Jones, D., Allen, C.R., Vernon, K.G.*, et al.* (2000). Influence of Helicobacter pylori on reactive oxygen-induced gastric epithelial cell injury. *Carcinogenesis* **21,** 2091-2095.

Sokolova, O., Bozko, P.M. and Naumann, M. (2008). Helicobacter pylori suppresses glycogen synthase kinase 3beta to promote beta-catenin activity. *J Biol Chem* **283,** 29367-29374.

Sokolova, O., Vieth, M. and Naumann, M. (2012). Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in Helicobacter pylori infection. *Gut*.

Stylli, S.S., Stacey, T.T., Verhagen, A.M., Xu, S.S., Pass, I., Courtneidge, S.A. and Lock, P. (2009). Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. *J Cell Sci* **122,** 2727-2740.

Su, B., Johansson, S., Fallman, M., Patarroyo, M., Granstrom, M. and Normark, S. (1999). Signal transduction-mediated adherence and entry of Helicobacter pylori into cultured cells. *Gastroenterology* **117,** 595-604.

Suerbaum, S. and Michetti, P. (2002). Helicobacter pylori infection. *N Engl J Med* **347,** 1175-1186.

Suriano, G., Mulholland, D., de Wever, O., Ferreira, P., Mateus, A.R., Bruyneel, E.*, et al.* (2003a). The intracellular E-cadherin germline mutation V832 M lacks the ability to mediate cell-cell adhesion and to suppress invasion. *Oncogene* **22,** 5716-5719.

Suriano, G., Oliveira, M.J., Huntsman, D., Mateus, A.R., Ferreira, P., Casares, F.*, et al.* (2003b). E-cadherin germline missense mutations and cell phenotype: evidence for the independence of cell invasion on the motile capabilities of the cells. *Hum Mol Genet* **12,** 3007-3016.

Suzuki, M., Mimuro, H., Kiga, K., Fukumatsu, M., Ishijima, N., Morikawa, H.*, et al.* (2009). Helicobacter pylori CagA phosphorylation-independent function in epithelial proliferation and inflammation. *Cell Host Microbe* **5,** 23-34.

Suzuki, M., Mimuro, H., Suzuki, T., Park, M., Yamamoto, T. and Sasakawa, C. (2005). Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion. *J Exp Med* **202,** 1235-1247.

Tabassam, F.H., Graham, D.Y. and Yamaoka, Y. (2008). OipA plays a role in Helicobacter pylori-induced focal adhesion kinase activation and cytoskeletal re-organization. *Cell Microbiol* **10,** 1008-1020.

Tabassam, F.H., Graham, D.Y. and Yamaoka, Y. (2009). Helicobacter pylori activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. *Cell Microbiol* **11,** 70-82.

Tabassam, F.H., Graham, D.Y. and Yamaoka, Y. (2011). Paxillin is a novel cellular target for converging Helicobacter pylori-induced cellular signaling. *Am J Physiol Gastrointest Liver Physiol* **301,** G601-611.

Takaishi, S. and Wang, T.C. (2007). Gene expression profiling in a mouse model of Helicobacter-induced gastric cancer. *Cancer Sci* **98,** 284-293.

Takeichi, M. (1991). Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251,** 1451-1455.

Tammer, I., Brandt, S., Hartig, R., Konig, W. and Backert, S. (2007). Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. *Gastroenterology* **132,** 1309-1319.

Tan, S., Tompkins, L.S. and Amieva, M.R. (2009). Helicobacter pylori usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog* **5,** e1000407.

Tao, R., Hu, M.F., Lou, J.T. and Lei, Y.L. (2007). Effects of H pylori infection on gap-junctional intercellular communication and proliferation of gastric epithelial cells in vitro. *World J Gastroenterol* **13,** 5497-5500.

Tegtmeyer, N., Wittelsberger, R., Hartig, R., Wessler, S., Martinez-Quiles, N. and Backert, S. (2011). Serine phosphorylation of cortactin controls focal adhesion kinase activity and cell scattering induced by Helicobacter pylori. *Cell Host Microbe* **9,** 520-531.

Tegtmeyer, N., Zabler, D., Schmidt, D., Hartig, R., Brandt, S. and Backert, S. (2009). Importance of EGF receptor, HER2/Neu and Erk1/2 kinase signalling for host cell elongation and scattering induced by the Helicobacter pylori CagA protein: antagonistic effects of the vacuolating cytotoxin VacA. *Cell Microbiol* **11,** 488-505.

Terakado, M., Gon, Y., Sekiyama, A., Takeshita, I., Kozu, Y., Matsumoto, K.*, et al.* (2011). The Rac1/JNK pathway is critical for EGFR-dependent barrier formation in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* **300,** L56-63.

Teramoto, T., Onda, M., Tokunaga, A. and Asano, G. (1996). Inhibitory effect of anti-epidermal growth factor receptor antibody on a human gastric cancer. *Cancer* **77,** 1639-1645.

Terres, A.M., Pajares, J.M., O'Toole, D., Ahern, S. and Kelleher, D. (1998). H pylori infection is associated with downregulation of E-cadherin, a molecule involved in epithelial cell adhesion and proliferation control. *J Clin Pathol* **51,** 410-412.

Thorns, V., Walter, G.F. and Thorns, C. (2003). Expression of MMP-2, MMP-7, MMP-9, MMP-10 and MMP-11 in human astrocytic and oligodendroglial gliomas. *Anticancer Res* **23,** 3937-3944.

Toiyama, Y., Yasuda, H., Saigusa, S., Matushita, K., Fujikawa, H., Tanaka, K.*, et al.* (2012). Co-expression of hepatocyte growth factor and c-Met predicts peritoneal dissemination established by autocrine hepatocyte growth factor/c-Met signaling in gastric cancer. *Int J Cancer* **130,** 2912-2921.

Tombola, F., Morbiato, L., Del Giudice, G., Rappuoli, R., Zoratti, M. and Papini, E. (2001). The Helicobacter pylori VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J Clin Invest* **108,** 929-937.

Trusolino, L., Bertotti, A. and Comoglio, P.M. (2001). A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. *Cell* **107,** 643-654.

Tsai, S.Y., Huang, Y.L., Yang, W.H. and Tang, C.H. (2012). Hepatocyte growth factor-induced BMP-2 expression is mediated by c-Met receptor, FAK, JNK, Runx2, and p300 pathways in human osteoblasts. *Int Immunopharmacol*.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* **2,** 285-293.

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M. and Hatakeyama, M. (2003). Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J Biol Chem* **278,** 3664-3670.

Tsutsumi, R., Takahashi, A., Azuma, T., Higashi, H. and Hatakeyama, M. (2006). Focal adhesion kinase is a substrate and downstream effector of SHP-2 complexed with Helicobacter pylori CagA. *Mol Cell Biol* **26,** 261-276.

Turnberg, L.A. (1985). Gastric mucosal defence mechanisms. *Scand J Gastroenterol Suppl* **110,** 37-40.

Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T. and Kitamura, N. (1995). Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373,** 702-705.

Uemura, N., Mukai, T., Okamoto, S., Yamaguchi, S., Mashiba, H., Taniyama, K.*, et al.* (1997). Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **6,** 639-642.

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M.*, et al.* (2001). Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med* **345,** 784-789.

Ukropec, J.A., Hollinger, M.K., Salva, S.M. and Woolkalis, M.J. (2000). SHP2 association with VE-cadherin complexes in human endothelial cells is regulated by thrombin. *J Biol Chem* **275,** 5983-5986.

van der Geer, P., Hunter, T. and Lindberg, R.A. (1994). Receptor protein-tyrosine kinases and their signal transduction pathways. *Annual review of cell biology* **10,** 251-337.

van Doorn, L.J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E.K.*, et al.* (1998). Expanding allelic diversity of Helicobacter pylori vacA. *J Clin Microbiol* **36,** 2597-2603.

van Hengel, J., Vanhoenacker, P., Staes, K. and van Roy, F. (1999). Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. *Proc Natl Acad Sci U S A* **96,** 7980-7985.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P.*, et al.* (2004). Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. *Nat Immunol* **5,** 1166-1174.

Visse, R. and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* **92,** 827-839.

Vleminckx, K., Vakaet, L., Jr., Mareel, M., Fiers, W. and van Roy, F. (1991). Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell* **66,** 107-119.

Wallasch, C., Crabtree, J.E., Bevec, D., Robinson, P.A., Wagner, H. and Ullrich, A. (2002). Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. *Biochem Biophys Res Commun* **295,** 695-701.

Watanabe, T., Tada, M., Nagai, H., Sasaki, S. and Nakao, M. (1998). Helicobacter pylori infection induces gastric cancer in mongolian gerbils. *Gastroenterology* **115,** 642-648.

Weidner, K.M., Behrens, J., Vandekerckhove, J. and Birchmeier, W. (1990). Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. *J Cell Biol* **111,** 2097-2108.

Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J. and Birchmeier, W. (1996). Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* **384,** 173-176.

Wells, A., Ware, M.F., Allen, F.D. and Lauffenburger, D.A. (1999). Shaping up for shipping out: PLCgamma signaling of morphology changes in EGF-stimulated fibroblast migration. *Cell Motil Cytoskeleton* **44,** 227-233.

Wessler, S., Hocker, M., Fischer, W., Wang, T.C., Rosewicz, S., Haas, R.*, et al.* (2000). Helicobacter pylori activates the histidine decarboxylase promoter through a mitogen-activated protein kinase pathway independent of pathogenicity island-encoded virulence factors. *J Biol Chem* **275,** 3629-3636.

Wex, T., Kuester, D., Monkemuller, K., Stahr, A., Fry, L.C., Kandulski, A.*, et al.* Assessment of desmosomal components (desmoglein 1-3, plakoglobin) in cardia mucosa in relation to gastroesophageal reflux disease and Helicobacter pylori infection. *Hum Pathol*.

Weydig, C., Starzinski-Powitz, A., Carra, G., Lower, J. and Wessler, S. (2007). CagA-independent disruption of adherence junction complexes involves E-cadherin shedding and implies multiple steps in Helicobacter pylori pathogenicity. *Exp Cell Res* **313,** 3459-3471.

Wiedemann, T., Hofbaur, S., Tegtmeyer, N., Huber, S., Sewald, N., Wessler, S.*, et al.* (2012). Helicobacter pylori CagL dependent induction of gastrin expression via a novel alphavbeta5-integrin-integrin linked kinase signalling complex. *Gut* **61,** 986-996.

Wilkins-Port, C.E. and Higgins, P.J. (2007). Regulation of extracellular matrix remodeling following transforming growth factor-beta1/epidermal growth factor-stimulated epithelial-mesenchymal transition in human premalignant keratinocytes. *Cells Tissues Organs* **185,** 116-122.

Wilkinson, S.M., Uhl, J.R., Kline, B.C. and Cockerill, F.R., 3rd (1998). Assessment of invasion frequencies of cultured HEp-2 cells by clinical isolates of Helicobacter pylori using an acridine orange assay. *J Clin Pathol* **51,** 127-133.

Windle, H.J. and Kelleher, D. (1997). Identification and characterization of a metalloprotease activity from Helicobacter pylori. *Infect Immun* **65,** 3132-3137.

Windsor, L.J., Grenett, H., Birkedal-Hansen, B., Bodden, M.K., Engler, J.A. and Birkedal-Hansen, H. (1993). Cell type-specific regulation of SL-1 and SL-2 genes. Induction of the SL-2 gene but not the SL-1 gene by human keratinocytes in response to cytokines and phorbolesters. *J Biol Chem* **268,** 17341-17347.

Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S. and Vogelstein, B. (1992). Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* **89,** 2965-2969.

Wong, B.C., Lam, S.K., Wong, W.M., Chen, J.S., Zheng, T.T., Feng, R.E*., et al.* (2004). Helicobacter pylori eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA* **291,** 187-194.

Wong, B.C., Wang, W.P., So, W.H., Shin, V.Y., Wong, W.M., Fung, F.M*., et al.* (2001). Epidermal growth factor and its receptor in chronic active gastritis and gastroduodenal ulcer before and after Helicobacter pylori eradication. *Alimentary pharmacology & therapeutics* **15,** 1459-1465.

Wroblewski, L.E., Noble, P.J., Pagliocca, A., Pritchard, D.M., Hart, C.A., Campbell, F*., et al.* (2003). Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. *J Cell Sci* **116,** 3017-3026.

Wroblewski, L.E., Shen, L., Ogden, S., Romero-Gallo, J., Lapierre, L.A., Israel, D.A*., et al.* (2009). Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. *Gastroenterology* **136,** 236-246.

Wu, C.Y., Wang, C.J., Tseng, C.C., Chen, H.P., Wu, M.S., Lin, J.T*., et al.* (2005). Helicobacter pylori promote gastric cancer cells invasion through a NF-kappaB and COX-2-mediated pathway. *World J Gastroenterol* **11,** 3197-3203.

Wu, J.Y., Lu, H., Sun, Y., Graham, D.Y., Cheung, H.S. and Yamaoka, Y. (2006). Balance between polyoma enhancing activator 3 and activator protein 1 regulates Helicobacter pylori-stimulated matrix metalloproteinase 1 expression. *Cancer Res* **66,** 5111-5120.

Wu, S., Lim, K.C., Huang, J., Saidi, R.F. and Sears, C.L. (1998). Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci U S A* **95,** 14979-14984.

Xiang, Z., Censini, S., Bayeli, P.F., Telford, J.L., Figura, N., Rappuoli, R. and Covacci, A. (1995). Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* **63,** 94-98.

Xu, C.X., Jia, Y., Yang, W.B., Wang, F. and Shen, S.R. (2008). [Relationship between Helicobacter pylori infection and expression of connexin (Cx) 32 and Cx43 genes in gastric cancer and gastric precancerous lesions]. *Zhonghua Yi Xue Za Zhi* **88,** 1523-1527.

Xu, K.P. and Yu, F.S. (2007). Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. *Investigative ophthalmology & visual science* **48,** 2242-2248.

Yamaoka, Y. (2011). Mechanisms of disease: Helicobacter pylori virulence factors. *Nat Rev Gastroenterol Hepatol* **7,** 629-641.

Yan, F., Cao, H., Chaturvedi, R., Krishna, U., Hobbs, S.S., Dempsey, P.J*., et al.* (2009). Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. *Gastroenterology* **136,** 1297-1307, e1291-1293.

Yanagisawa, M. and Anastasiadis, P.Z. (2006). p120 catenin is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness. *J Cell Biol* **174,** 1087-1096.

Yanagisawa, N., Geironson, L., Al-Soud, W.A. and Ljungh, S. (2005). Expression of matrix metalloprotease-2, -7 and -9 on human colon, liver and bile duct cell lines by enteric and gastric Helicobacter species. *FEMS immunology and medical microbiology* **44,** 197-204.

Yap, A.S., Niessen, C.M. and Gumbiner, B.M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J Cell Biol* **141,** 779-789.

Yarden, Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer* **37 Suppl 4,** S3-8.

Yarden, Y. and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* **2,** 127-137.

Yin, Y., Grabowska, A.M., Clarke, P.A., Whelband, E., Robinson, K., Argent, R.H.*, et al.* (2010). Helicobacter pylori potentiates epithelial:mesenchymal transition in gastric cancer: links to soluble HB-EGF, gastrin and matrix metalloproteinase-7. *Gut* **59,** 1037-1045.

Yokoyama, T., Otani, Y., Kurihara, N., Sakurai, Y., Kameyama, K., Suzuki, H.*, et al.* (2000). Matrix metalloproteinase expression in cultured human gastric wall fibroblasts--interactions with Helicobacter pylori isolated from patients with ulcers. *Alimentary pharmacology & therapeutics* **14 Suppl 1,** 193-198.

Yokozaki, H. (2000). Molecular characteristics of eight gastric cancer cell lines established in Japan. *Pathol Int* **50,** 767-777.

Zenmyo, M., Hiraoka, K., Sasaguri, T., Komiya, S., Inoue, A., Morimatsu, M. and Sasaguri, Y. (1996). Interleukin-4 stimulates rheumatoid synovial fibroblasts to express matrix metalloproteinase-1 (tissue collagenase) and histamine H1 receptor mRNA. *Biochemistry and molecular biology international* **40,** 1253-1260.

Zheng, L., Wang, L., Ajani, J. and Xie, K. (2004). Molecular basis of gastric cancer development and progression. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association* **7,** 61-77.

Zhu, W., Leber, B. and Andrews, D.W. (2001). Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. *EMBO J* **20,** 5999-6007.

Zhuang, X., Lin, S., Zheng, J., Wang, L., Sun, G. and Li, Y. (2001). [The prognostic research of expression and relationship between Helicobacter pylori of c-met oncogene correlation with gastric mucosal proliferation]. *Zhonghua Nei Ke Za Zhi* **40,** 381-384.

Zullo, A., Romiti, A., Borrini, F., Hassan, C., Stella, F., Sarcina, I.*, et al.* (2004). Alteration of E-cadherin expression in gastric mucosa: role of intestinal metaplasia and Helicobacter pylori infection. *Anticancer Res* **24,** 1603-1607.

SUMMARY

H. pylori is a gram-negative bacterium that infects more than half of the human population, causing chronic superficial gastritis, that can progress to more severe diseases, including gastric carcinoma.

H. pylori induces alterations in host cell signaling pathways that result in alterations of the normal cell behavior. One of the less explored phenotypes associated with *H. pylori* infection is cell invasion, a phenotype which involves a multitude of processes including cell migration and extracellular matrix degradation by MMPs (Matrix Metalloproteinases). The general aim of the work presented in this thesis was to evaluate the role of *H. pylori* infection in the induction of gastric epithelial cell invasion. The initial approach consisted on the evaluation of the invasive capacity through extracellular matrix components of the non-invasive human gastric cancer cell line AGS after infection with *H. pylori*. The role of the bacterial virulence factors CagA, T4SS (Type 4 Secretion System), and VacA was also evaluated. After establishing that the c-Met receptor was important for *H. pylori*-mediated AGS cell invasion, the role of the downstream targets of c-Met was also elucidated. Results showed that *H. pylori* strains with a competent T4SS induce host gastric cell invasion through extracellular matrix components via c-Met receptor-dependent Nck, PLC-γ, and c-Src activation.

Based on the aforementioned results pointing to a role of *H. pylori* in inducing extracellular matrix degradation by host cells, and since MMPs are important for extracellular matrix degradation, the next aim was to evaluate the role of *H. pylori* in MMP expression. Studies started with the validation of a panel of MMPs shown to be up-regulated in a cDNA expression microarray performed by our Group. After confirmation that *H. pylori* increased the expression of MMP-1, MMP-7, and MMP-10, a more detailed investigation on the effect of *H. pylori* on MMP-10 modulation was performed. Quantitative real-time polymerase chain reaction, western blot, enzymatic activity, and invasion assays were used in uninfected and *H. pylori*-infected cells. The role of the *H. pylori* virulence factors CagA and T4SS was addressed using bacterial mutants and a panel of *H. pylori* clinical isolates with known cagA status. The involvement of specific host cell signaling pathways and of receptor tyrosine kinases in modulation of MMP-10 expression by *H. pylori* was evaluated in experiments with siRNA gene silencing and with chemical inhibitors. Results obtained in Part I.2 of the Results of the thesis showed that MMP-10 expression is induced in gastric epithelial cells by CagA-positive *H. pylori* strains *via* the c-Met and EGF receptors, in a process that involves Nck and c-Src and also the ERK and JNK pathways, as well as it involvement in *H. pylori*-mediated cell invasion.

Since the E-cadherin-catenin complex functions as an invasion suppressor, the next aim was to evaluate whether the complex had a role in *H. pylori*-mediated cell invasion and signaling. For that, gastric cell lines with intact E-cadherin-catenin complex were used. Also, and for the purpose of comparison with previous data obtained in the AGS background, an AGS cell line with a wild-type E-cadherin was established. For the characterization of the structure and function of the adherens junctions, immunofluorescence, confocal microscopy and aggregation assays were used. The phosphorylation and interactions between proteins of the adherens junctions were evaluated by co-immunoprecipitation and western blot. The invasion capacity of the different cell lines was assessed with Matrigel assays. Results presented in this part of the thesis evidence that *H. pylori* infection leads to formation of a multiproteic complex composed by CagA, c-Met, E-cadherin, and p120-catenin. This complex impairs *H. pylori*-mediated c-Met and p120-catenin tyrosine phosphorylation and suppresses the invasive phenotype induced by *H. pylori*.

Altogether, these results suggest that the interaction between *H. pylori* and gastric epithelial cells leads to activation of host signaling pathways, modification of cellular functions, and induction of cell phenotypes which underlie disease, namely gastric carcinoma. The elucidation of *H. pylori*-host interactions may provide further insights on *H. pylori* pathogenesis and on the mechanisms relevant to gastric disease development.

SUMÁRIO

H. pylori é uma bactéria gram-negativa que infecta mais de metade da população humana, causando gastrite superficial crónica que pode progredir para doenças mais graves, incluindo o carcinoma gástrico.

H. pylori induz alterações nas vias de sinalização da célula do hospedeiro que resultam em alterações no comportamento normal da célula. Um dos fenótipos menos explorados associados com a infecção por *H. pylori* é a invasão celular, um fenótipo, que envolve uma multiplicidade de processos, incluindo a migração celular e a degradação da matriz extracelular por MMPs (Metaloproteases da Matriz). O objectivo geral do trabalho apresentado nesta tese foi o de avaliar o papel da infecção por *H. pylori* na indução de invasão das células epiteliais gástricas. A abordagem inicial consistiu na avaliação da capacidade invasiva através de componentes da matriz extracelular de células AGS, uma linha celular gástrica humana não-invasiva, após a infecção com *H. pylori*. O papel do fatores de virulência bacterianos CagA, T4SS (Sistema de Secreção Tipo 4) e VacA também foi avaliado. Depois de estabelecer que o receptor c-Met era importante para invasão celular das células AGS, mediada por *H. pylori,* o papel dos alvos a jusante do c-Met foi estudado. Os resultados mostraram que as estirpes de *H. pylori* com um T4SS competente induzem invasão de células gástricas do hospedeiro através dos componentes da matriz extracelular, por uma via dependente da activação de c-Met, Nck, PLC-γ, e c-Src.

Com base nos resultados acima, que apontam para um papel de *H. pylori* na indução de degradação da matriz extracelular das células do hospedeiro, e uma vez que as MMPs são importantes para a degradação da matriz extracelular, o objectivo seguinte foi o de avaliar o papel de *H. pylori* na expressão de MMPs. O estudo começou com a validação de um painel de MMPs sobre-expressas num *microarray* de expressão de cDNA realizado pelo Grupo. Após a confirmação de que a infecção por *H. pylori* aumentou a expressão das MMP-1, MMP-7 e MMP-10, foi investigado mais detalhadamente o efeito de *H. pylori* na modulação da MMP-10. Em células não infectadas e infectadas por *H. pylori* foram feitos ensaios de real-time-PCR, western blot, ensaios de actividade enzimática e invasão. O papel dos factores de virulência CagA e T4SS de *H. pylori* foi estudado utilizando estirpes mutantes e um painel de isolados clínicos de *H. pylori,* com o *status* do *cagA* conhecido. O envolvimento de vias de sinalização celular do hospedeiro e de receptores tirosina-cinase na modulação da expressão de MMP-10 por *H. pylori* foi avaliado em experiências com siRNA e com inibidores químicos. Os resultados obtidos na Parte I.2 dos Resultados mostraram que

a expressão de MMP-10 é induzida em células epiteliais gástricas por estirpes de *H. pylori* CagA-positivas, através dos receptores c-Met e EGFR, num processo que envolve as proteínas Nck e c-Src e também as vias de sinalização ERK e JNK, bem como o seu envolvimento na invasão celular mediada por *H. pylori.*

Uma vez que o complexo E-caderina-cateninas funciona como supressor de invasão, o objectivo seguinte foi o de avaliar o papel do complexo na invasão e sinalização celulares mediada por *H. pylori*. Para isso, foram utilizados linhas celulares gástricas com o complexo E-caderina-cateninas intacto. Além disso, e para efeitos de comparação com os dados anteriores obtidos com as células AGS, foi estabelecida uma linha celular AGS com expressão de E-caderina de tipo selvagem. Para a caracterização da estrutura e função das junções de adesão foi utilizada imunofluorescência, microscopia confocal e ensaios de agregação. A fosforilação e as interacções entre proteínas das junções de adesão foram avaliados por co-imunoprecipitação e western blot. A capacidade de invasão das diferentes linhas celulares foi avaliada com ensaios de Matrigel. Os resultados apresentados nesta parte da tese mostraram que a infecção por *H. pylori* leva à formação de um complexo multiproteico composto por CagA, c-Met, E-caderina e catenina p120 . Este complexo impede a fosforilação de residuos tirosina no c-Met e catenina p120 mediada por *H. pylori* e suprime o fenótipo invasivo induzido pela bacteria.

No seu conjunto, estes resultados sugerem que a interacção entre *H. pylori* e as células epiteliais gástricas conduz à activação de vias de sinalização do hospedeiro, à modificação de funções, e à indução de fenótipos celulares que poderão estar subjacentes ao desenvolvimento de doença, nomeadamente de carcinoma gástrico. A elucidação das interações *H. pylori*-hospedeiro pode fornecer informações sobre a patogénese de *H. pylori,* e sobre possíveis mecanismos relevantes para o desenvolvimento de doenças gástricas associadas à infecção.

PAPERS

Paper I

Helicobacter pylori CagA activates matrix metalloproteinase-10 in gastric epithelial cells through ERK and JNK-mediated pathways

Costa AM, Ferreira RM, Oliveira MJ, Carneiro F, Leite M, Figueiredo C.

In preparation

Helicobacter pylori CagA activates matrix metalloproteinase-10 in gastric epithelial cells through ERK and JNK-mediated pathways

Angela Margarida Costa[1,2], Rui Manuel Ferreira[1,2], Maria Jose Oliveira[3], Fatima Carneiro[1,2,4], Marina Leite[1], and Ceu Figueiredo[1,2]

[1]Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP); [2]Department of Pathology and Oncology, Faculty of Medicine of the University of Porto; [3]Institute of Biomedical Engineering (INEB); [4]Department of Pathology, Centro Hospitalar S. João, Porto, Portugal

Running Title: MMP-10 expression in response to *H. pylori*

ABSTRACT

Helicobacter pylori colonizes the human stomach inducing chronic gastritis, that in some cases evolves to more severe diseases, such as peptic ulcer disease and gastric carcinoma.

H. pylori infection leads to up-regulation of the expression and activity of several matrixmetalloproteinases (MMPs), both in gastric cell lines and in the gastric mucosa. The aim of this study was to analyze the mechanisms leading to up-regulation of MMP-10 in gastric epithelial cells induced by *H. pylori*.

Infection of AGS cells with *H. pylori* led to an increase in MMP-10 mRNA, protein secretion, and activity. *H. pylori* strains that were mutant for *cagA* or for *cagE* (lacking the ability to translocate CagA) failed to increase MMP-10 expression. These results were confirmed in cocultures of cells performed with a panel of *H. pylori* clinical isolates with known *cagA* status.

Treatment of AGS cells with HGF or EGF led to an increase in MMP-10 expression. Inhibition of c-Met, EGFR, and their common downstream targets Nck, and c-Src, with siRNAs or with chemical inhibitors abrogated *H. pylori*-induced MMP-10 expression, suggesting their involvement in MMP-10 up-regulation induced by the infection. Specific inhibitors of ERK1/2 and JNK abolished or significantly decreased *H. pylori*-induced MMP-10 expression, whereas an inhibitor of p38 enhanced MMP-10 expression. Inhibition of MMP-10 expression led to a decrease in gastric cell invasion mediated by *H. pylori* infection.

In conclusion, MMP-10 expression is stimulated by *H. pylori* strains containing CagA, in a process that involves c-Met and EGFR and their downstream targets Nck, and c-Src, *via* the ERK1/2 and JNK pathways. Induction of MMP-10 is also implicated in *H. pylori*-mediated cell invasion.

Keywords: *Helicobacter pylori*, MMP-10, CagA

INTRODUCTION

H. pylori are gram negative bacteria that infect the stomach of half of the world's population. Infection is associated with chronic gastritis (54), which in the majority of the cases remains for the lifetime of the host. However, some individuals may develop more severe disease such as peptic ulcer disease, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma (54, 67). The differences in clinical outcomes associated with *H. pylori* infection are likely the result of the interplay between host, environmental, and bacterial virulence factors.

About 60% of *H. pylori* strains contain a *cag* pathogenicity island (PAI) that encodes components of a type IV secretion system (T4SS). It has been shown that individuals infected with *H. pylori cag* PAI-positive strains have more severe gastric inflammation, and are more likely to develop peptic ulcer disease, and gastric carcinoma than individuals infected with *cag* PAI-negative strains (5, 13, 17, 21, 55). This is likely to occur since *H. pylori cag* PAI-positive are more interactive with the host cells than are *cag* PAI-negative strains, and induce stress response kinases such as ERK 1/2, JNK, and p38, and transcription factor NF-kB and AP-1 activation (29, 45, 76). As a consequence, *H. pylori* leads to increased expression of inflammatory cytokines such as IL-8, IL-1β, or TNF-α, cytoskeleton rearrangements, and cell motogenic and invasive phenotypes (11, 12, 44, 49), which may ultimately result in tissue damage in the gastric mucosa.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that degrade components of the extracellular matrix and have an central role in tissue remodeling and cell invasion (8). Additionally, MMPs cleave non-matrix targets including cell surface receptors, cytokines, cell-cell adhesion molecules, and other proteinases (65). MMPs participate in processes, such as cell proliferation, differentiation, adhesion, migration, angiogenesis, apoptosis, and inflammation, both in physiological and in pathological settings (8, 35).

The interaction between *H. pylori* and the gastric mucosa induces a variety of MMPs, including MMP-1, MMP-2, MMP-3, MMP-7, and MMP-9, directly or indirectly *via* induction of cytokine synthesis (4, 22, 33, 49, 57, 73).

cDNA microarray studies to investigate changes in gene expression in hypergastrinemic transgenic (INS-GAS) mice stomachs infected with *H. felis* (68), and in a mouse gastric epithelial progenitor cell line infected with a chronic atrophic gastritis-derived *H. pylori* strain (19), have suggested that *Helicobacter* up-regulates MMP-10. However, the modulation of MMP-10 by *H. pylori* has never been explored.

In addition, MMP-10 is overexpressed in several epithelial-derived cancers (6, 20, 38, 52, 71) and has been considered as marker of poor prognosis in gastric carcinoma patients (2, 36).

Here, we report that *H. pylori* stimulates the expression, protein secretion, and activity of MMP-10 in AGS cells, in a process dependent on the presence of CagA, and mediated by the receptor tyrosine kinases c-Met and EGFR, and its downstream targets Nck, c-Src, ERK1/2, and JNK. We further demonstrate that MMP-10 is involved in *H. pylori*-mediated gastric cell invasion.

MATERIALS AND METHODS

Cell culture and reagents

AGS cells derived from a human gastric carcinoma (ATCC, Rockville, MD), were maintained in RPMI 1640 (Gibco; Grand Island, NY, USA) with 10% fetal bovine serum (Hyclone; Cramlington, UK), 200 µg/ml streptomycin, and 200 IU/ml penicillin (Gibco) at 37ºC, under a humidified atmosphere containing 5% CO2.

Bacterial strains and growth conditions

Bacteria were grown in tryptic soy agar supplemented with 5% sheep blood (BD Bioscience; Heidelberg, Germany) and incubated 48 hours at 37ºC under a microaerophilic atmosphere. *H. pylori* strains 26695 (ATCC 700392), Tx30a (ATCC 51932), 84183 and its *cagA* (84183Δ*cagA*) and *cagE* (84183Δ*cagE*) mutants (a kind gift from Prof. John Atherton), were used. A panel of *H. pylori* isolates with known *cagA*-status was also used. The presence of *cagA* was determined by PCR-reverse hybridization as previously described (74).

Infection of gastric cells

80% confluent cell monolayers were washed in PBS and incubated in antibiotic-free medium. For infection, *H. pylori* was added to cells at a multiplicity of infection (MOI) of 100. Cultures were maintained at 37ºC under a 5% CO2 atmosphere for 24 hours. After infection, conditioned medium was collected, and the cells were lysed for RNA isolation.

Quantification of mRNA by Real-Time PCR

Total RNA was extracted using TriPure Isolation Reagent (Roche; Mannheim, Germany). One µg of RNA was used for cDNA synthesis, using Random Primers and SuperScript® II Reverse Transcriptase (Invitrogen; Carlsbad, CA, USA). Real-time PCR was performed using a TaqMan Mix (Applied Biosystems; Branchburg, NJ, USA) and the MMP-10 TaqMan probe (Hs00233987_m1, Applied Biosystems). Relative MMP-10 expression was normalized to levels of GAPDH (Human GAPDH Endogenous Control (FAM™ Dye / MGB Probe, Non-Primer Limited), Applied Biosystems). PCR was performed in a 7500 Real Time PCR System (Applied Biosystems).

Preparation of Conditioned Medium

Conditioned medium was collected, centrifuged at 3824 g for 3 min and filtered through 0.2 µm pore size filters (Advantec). To measure MMP-10 activity, conditioned medium

was concentrated using a Speed-Vac system. For SDS-PAGE, conditioned medium was concentrated using Amicon® ultra-0.5 centrifugal filter devices, 10 kDa cutoff (Millipore; Billerica, MA, USA).

Preparation of cell lysates

Cells were lysed in Catenin lysis buffer (1% Triton X-100, 1% NP-40 in PBS, pH 7.4) with proteases and phosphatases inhibitors (3mM sodium vanadate, 20mM NaF, 1mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10μg/ml leupeptin). 25 μg of protein were used for analysis of total lysates. Proteins were separated by SDS-PAGE.

Immunoblot analysis

Proteins were transferred onto Hybond nitrocellulose membranes (GE Healthcare; Little Chalfont, Buckinghamshire, UK), blocked with 5% non-fat milk in PBS +0.1% Twen-20 (PBS-T) and incubated overnight at 4ºC with primary antibody. It was used an anti MMP-10 antibody (Santa Cruz Biotechnology: Santa Cruz, CA, USA), and a HRP-conjugated donkey anti-goat (Santa Cruz Biotechnology). Subsequently Luminata™ Forte Western HRP Substract (Millipore) detection was performed.

MMP-10 activity

MMP-10 activity in conditioned medium was assessed using the fluorimetric assay SensoLyte® 520 MMP-10 Assay Kit (AnaSpec; Fremont, CA, USA), according to the manufacturer's instructions. Recombinant MMP-10 (AnaSpec) was used to do a standard curve of concentrations and a fluorigenic FAM/QXL™ 520 FRET peptide was used as a substrate.

Cell treatment

Pharmacological inhibitors U-0126, SB203580, SP600125, and AG-1478 were obtained from Cayman Chemicals (Ann Arbor, MI, USA), and PP2 from Calbiochem (Gibbstown, NJ, USA). The concentrations of inhibitors used were 25 μM for U-0126, 20 μM for SB203580 and SP600125 and 5μM for PP2 and AG-1478. Cells were treated one hour before the infection, and the inhibitor was not removed during the experiment. HGF at 250 ng/mL (Immunotools; Friesoythe, Germany) and EGF at 50 ng/mL (Sigma; Steinheim, Germany) were used to treat the cells for 24hours.

Small interference RNA (siRNA) transfection

50% confluent monolayers were washed and incubated in serum- and antibiotic-free medium. siRNAs targeting c-Met, Nck, PLC-γ, c-Src, MMP-10, and Non-silencing

siRNA were obtained from Qiagen. Transfection was performed using Lipofectamine 2000 (Invitrogen). Silencing efficiencies of siRNAs were evaluated, 48 hours after transfection, by western blot for all siRNAs, except for MMP-10, which was evaluated by Real-time PCR.

Invasion assays

Matrigel-coated 24-well invasion inserts of 8 µm pore-size filters (BD Biosciences) were incubated one hour at 37ºC with antibiotic-free medium. After this period, $5x10^4$ cells, in the presence or absence of *H. pylori* at a MOI of 100, were incubated 24 hours at 37ºC. Filters were washed with PBS and fixed in 4% paraformaldehyde. Non-invading cells were stained and mounted in Vectashield with DAPI (Vector Laboratories; Burlingame, CA, USA). Invasive cells were scored in 20 microscopic fields (20X objective).

Statistical analysis

Data were analyzed with Student's t-test and expressed as mean values of at least three independent experiments ± standard errors. The comparison between MMP-10 expression and the *cagA* status of the *H. pylori* strains was performed using the non-parametric Mann-Whitney test. For that, a variable that represents the average of the assays for each strain was created, and strains were divided into *cagA*-positive and *cagA*-negative groups. Differences were considered significant at p values lower than 0.05.

RESULTS

H. pylori increases MMP-10 expression and activity

Increased expression of several MMPs has been reported in the context of *H. pylori* infection and in gastric carcinoma (2, 19, 33, 68). Specifically, the modulation of MMP-10 by *H. pylori* has never been explored in depth in human gastric epithelial cells. Therefore, we assessed the role of *H. pylori* infection in MMP-10 expression and activity. For that, AGS gastric cells were cultured in the presence or absence of *H. pylori* strain 26695 at a MOI of 100. After 24 hours of infection, RNA was isolated and MMP-10 mRNA levels were analyzed by quantitative real-time PCR (qRT-PCR). As shown in Figure 1A, *H. pylori* induced a 40-fold increase in MMP-10 mRNA levels compared to uninfected cells, which display very low levels of MMP-10 ($p < 0.001$).

Next, we tested whether the observed increase in MMP-10 mRNA expression was accompanied by an increase in MMP-10 secretion. For that, conditioned media of *H. pylori*-infected and uninfected AGS cells were collected and concentrated 2x, and the levels of secreted MMP-10 were detected by western blot. The results showed that *H. pylori* induced not only increased MMP-10 mRNA expression, but also induced secretion of MMP-10 (Figure 1B). No MMP-10 secretion was detected in AGS control cells. To further elucidate the activity of the secreted MMP-10, conditioned media of infected and uninfected AGS cells were analyzed using a fluorimetric activity assay. As it can be seen in Figure 1C, the MMP-10 secreted by AGS cells upon *H. pylori* infection was active.

H. pylori-mediated MMP-10 expression is CagA dependent

To define the relative contribution of the CagA and of the T4SS virulence factors to *H. pylori*-mediated increase in MMP-10 expression, AGS cells were infected for 24 hours with *H. pylori* strain 84183 and its *cagA* (84183*ΔcagA*) and T4SS (84183*ΔcagE*) mutants.

The analysis of MMP-10 mRNA levels by qRT-PCR showed that infection with *H. pylori* wild type 84183 and 26695 strains induce similar expression levels (Figures 1 A and 2A). In contrast, infection with the 84183*ΔcagA* and 84183*ΔcagE* mutants, lacking *cagA* and a functional T4SS, respectively, lead to the expression of significantly less amounts of MMP-10 (Figure 2A). These results show that the increase in MMP-10 mRNA induced by *H. pylori* is CagA-dependent.

To confirm these results in strains which have not been manipulated, we used *H. pylori* clinical isolates which have been submitted to low number of passages in the laboratory and for which the *cagA* status was known. AGS cells were infected with *H.*

pylori isolates and, in parallel, with the non-pathogenic strain Tx30a. After 24 hours of infection, the MMP-10 mRNA levels were quantified by qRT-PCR. Infection with *cagA*-positive strains led to a more pronounced increase in MMP-10 expression than did *cagA*-negative strains (Figure 2B). The main exception was strain CI-50 that although *cagA*-positive was not able to significantly up-regulate MMP-10 (Figure 2B). It is possible that this strain has an impaired T4SS and is not able to translocate CagA into the host cells. Curiously, Tx30a was the strain that among all *cagA*-negative strains induced a higher level of MMP-10, which was still much lower than the levels of MMP-10 induced by *cagA*-positive strains. Overall, these results point to an important role of CagA in MMP-10 up-regulation mediated by *H. pylori*.

H. pylori increases MMP-10 expression via c-Met and EGFR

It has been reported that MMPs may be up-regulated by growth factors that activate RTKs (16, 39). In fact, EGFR has been implicated in MMP-10 up-regulation in squamous cell carcinoma of head and neck (77). Interestingly, *H. pylori* infection has also been shown to activate EGFR and c-Met (11, 49, 75). Therefore, we addressed whether these two RTKs were implicated in *H. pylori*-mediated MMP-10 expression. First, we tested whether stimulation of AGS cells with HGF and EGF, the natural ligands of c-Met and EGFR respectively, altered MMP-10 expression. AGS cells were treated with HGF and EGF for 24 hours. The MMP-10 expression was analyzed by qRT- PCR. Both HGF and EGF enhanced MMP-10 expression, with EGF inducing a more pronounced increase (Figure 3A and 3B), demonstrating that MMP-10 can be up-regulated *via* c-Met and EGFR in AGS cells. Next, the role of c-Met and EGFR in the induction of MMP-10 during infection was addressed.

For that, AGS cells were knocked-down for c-Met by siRNA and for EGFR by the chemical inhibitor AG-1478, and infected or not with *H. pylori* for 24 hours. The downregulation of c-Met or EGFR abrogated *H. pylori*-mediated MMP-10 expression (Figure 3C and 3D). These findings indicate that the increase in MMP-10 expression induced by *H. pylori* is mediated by the activation of c-Met and EGFR.

c-Src and Nck, but not PLC-γ, are involved in H. pylori-induced MMP-10 expression

To further dissect the molecular mechanisms involved in c-Met and EGFR signaling that lead to MMP-10 up-regulation by *H. pylori*, we chose common c-Met and EGFR downstream targets described to be important for *H. pylori*-mediated signaling. To fulfill this aim, the expression of Nck, PLC-γ, and c-Src in AGS cells was downregulated using siRNAs. Efficient knockdown was achieved and was maximal 48 hours after

transfection. As control, AGS cells were transfected with a Non-silencing siRNA. Since the silencing of Src was difficult to achieve, the chemical inhibitor PP2 directed to the Src family kinases was also used. Cells were treated one hour before infection with PP2 or DMSO and then infected for 24 hours. RNA was isolated after this period and analyzed by qRT-PCR. As expected, MMP-10 expression increased in AGS cells infected with *H. pylori*, but this increase was attenuated in cells silenced for Nck (Figure 4A). Similarly *H. pylori*-mediated MMP-10 expression was decreased in cells silenced for Src or in which Src activity was inhibited with PP2 (Figure 4C and 4D). In contrast, the silencing of PLC-γ had no effect in *H. pylori*-mediated MMP-10 expression (Figure 4B). These results point to a role for Nck and Src in the regulation of MMP-10 during infection.

H. pylori increases MMP-10 expression through ERK and JNK, but not p38 pathways

Because ERK, JNK, and p38 pathways are involved in the induction of different MMPs (59, 60) and in *H. pylori*-driven signaling (45, 76), and because they can function downstream c-Met and EGFR receptors (7, 70, 72), we next determined their involvement in *H. pylori*-mediated MMP-10 expression. For this purpose, AGS cells were treated with chemical inhibitors of ERK1/2 (U-0126), JNK (SP600125), and p38 (SB203580), or with the vehicle (DMSO) alone, 1 hour before infection. Cells were then infected with *H. pylori* 26695 in the presence of the inhibitors for 24 hours, and MMP-10 expression were measured by qRT-PCR. Infection with *H. pylori* in the presence of DMSO did not affect MMP-10 expression stimulated by the bacteria alone (Figure 5A, 5B and 1A). The treatment with the ERK1/2 inhibitor resulted in complete abrogation of MMP-10 expression, whereas treatment with the JNK inhibitor led to a significant decrease of MMP-10 expression after *H. pylori* infection (Figure 5A). In contrast, the inhibition of p38 resulted in enhanced expression of MMP-10 after infection (Figure 5 B). Taken together, these results suggest that MMP-10 expression induced by *H. pylori* is partially mediated by JNK and ERK1/2 pathways, and that p38 may have an inhibitory role in this process.

MMP-10 promotes cell invasion mediated in *H. pylori* infection

As one of the main functions of MMPs is the turnover and remodeling of the extracellular matrix (53), and since it was previously reported that *H. pylori cag*-positive strains induce gastric cell invasion in a context of increased MMP expression and activity (49, 64), we next determined whether MMP-10 is involved in cell invasion mediated by *H. pylori*. AGS cells were transiently transfected with a siRNA abrogating

MMP-10 expression, and the efficiency of the knockdown was accessed by qRT-PCR. Invasion assays were performed 24 hours after transfection, by incubating AGS cells with *H. pylori* on Matrigel filters for an additional 24 hours. Silencing of MMP-10 inhibited *H. pylori*-induced MMP-10 expression in AGS cells (Figure 6A), and significantly inhibited cell invasion in response to *H. pylori* infection (Figure 6B). These findings demonstrate that MMP-10 plays a role in *H. pylori*-mediated cell invasion.

DISCUSSION

Persistent infection of the gastric mucosa with *H. pylori* and concomitant chronic inflammation underlies the association between this pathogen and peptic ulceration and gastric neoplasia. Despite the mechanisms through which *H. pylori* promotes ulcer and carcinoma have not been fully elucidated, the capability of this infectious organism to stimulate gastric epithelial cell secretion of MMPs, with possible induction of mucosal tissue damage by degradation of extracellular matrix components and by modulation of cytokine and chemokine activity, may partly explain the association.

Our data indicates that *H. pylori* infection of gastric epithelial cells stimulates the expression and secretion of MMP-10. MMP-10, also known as stromelysin-2, has been associated with a broad spectrum of pathological conditions, including inflammatory diseases and cancer (6, 20, 38, 47, 52, 62, 71). MMP-10 is up-regulated in lung, head and neck, esophagus, brain, and liver cancers (6, 20, 38, 52, 71). This MMP has been recently detected as one of the highly expressed genes in gastric carcinoma specimens compared with normal tissues by serial analysis of gene expression (SAGE) data analysis (2), and high levels of MMP-10 were found in serum samples from patients with gastric carcinoma (65/69, 94.2%). In these patients, MMP-10 has been considered as a marker of poor prognosis (2, 36). Nevertheless, and although MMP-10 expression has been associated with *Helicobacter* infections in cDNA expression microarrays (19, 33, 68), this is the first study in which MMP-10 regulation is addressed in the context of *H. pylori* infection.

Our data also indicates that up-regulation of MMP-10 expression by *H. pylori* is dependent on infection with CagA-positive strains. The CagA protein is a T4SS effector that is translocated into the host cells, where it can be phosphorylated by Src and Abl family kinases (46, 63, 69). Both the phosphorylated and the non-phosphorylated forms of CagA are able to activate multiple signaling pathways in the host cell, leading to cell proliferation, cytoskeletal rearrangements, and disruption of cell-cell junctions (1, 63). Consistent with the functional activities of CagA, infection with *H. pylori* strains that are CagA-positive results in more severe disease, including peptic ulcer disease and gastric carcinoma (5, 13, 17, 55).

Our results that c-Met and EGFR are associated with *H. pylori*-mediated MMP-10 expression are novel findings. It has been shown that MMPs may be up-regulated by growth factors that activate RTKs (16, 39). c-Met and EGFR are RTKs previously reported to be targeted by *H. pylori* (11, 75). In fact, c-Met is one of the CagA targets (11, 49, 50), in keeping with our results that Cag-A positive strains are significantly better inducers of MMP-10 than CagA-negative strains.

The EGFR was previously shown to be involved in MMP-10 up-regulation in squamous cell carcinoma of head and neck (78). Moreover, and in the context of *H. pylori* infection, EGF family members were shown to be involved in the stimulation of other MMPs, such as MMP-7, (79). On the other hand, *H. pylori*-mediated MMP up-regulation results in an increase of HB-EGF processing, a ligand of EGFR, and thus leads to an increase in the receptor activation (75, 81). Although apparently contradicting, these data may point to a feedback regulation mechanism mediated by MMPs during *H. pylori* infection. The fact that two RTKs are involved in the same cellular process may be an indication that they cooperate and compensate the absence of the other. This hypothesis is in accordance with previous descriptions of cross-talk between c-Met and EGFR (27, 40, 43, 80).

Nck, PLC-γ, and Src are all described to function downstream c-Met and EGFR (9, 24, 28, 32, 48, 58). Of these, only Nck and Src were shown to be involved in MMP-10 modulation by *H. pylori*. Nck is important for cytoskeletal re-organization, cell motility, and formation of invadopodia (51, 61, 66). In an infection context, Nck interacts with the enteropathogenic *Escherichia coli* virulence factor Tir, leading to actin pedestal formation necessary for bacteria internalization into the host cell (23). Here, we describe for the first time a role for Nck in the context of *H. pylori* infection.

The non-receptor tyrosine Src kinase is linked to cell invasion and proteolysis in several cancer models (10, 26, 34), and in some cases this occurs via c-Met activation (26), or by stimulation with cytokines (37). Src is also one of the kinases of CagA (63), which we found to be important in *H. pylori*-mediated MMP-10 expression. Whether the phosphorylation of CagA is necessary for MMP-10 up-regulation will be an interesting issue to address. Indeed, in a previous report the inhibition of CagA phosphorylation using a chemical inhibitor of Src resulted in a decrease of *H. pylori*-mediated MMP-9 secretion (42).

We have demonstrated that the ERK1/2 and JNK signaling pathways are involved in *H. pylori*-induced MMP-10 expression. These observations, together with the finding that EGFR is involved in *H. pylori* stimulation of MMP-10 are also consistent with previous reports of *H. pylori*-mediated ERK activation via transactivation of the EGFR (30, 31). The involvement ERK1/2, JNK, and p38 signal transduction pathways in MMP regulation has been previously described (59, 60), including in the context of *H. pylori* infection (14, 33, 42, 56). In contrast to ERK and JNK, our results showed that inhibition of p38 signaling significantly stimulated MMP-10 expression. Curiously, the finding that inhibition of p38 enhanced MMP-10 expression was also reported in head and neck cancer (15). Our results with *H. pylori* infection are similar to those obtained by Pillinger *et al.* using the same AGS cell line, and which detected a significantly

enhancing effect of the p38 inhibitor SB203580 on MMP-1 expression (55). Similar findings were also obtained in AGS cells by Nam *et al*. in which p38 inhibitor slightly activated MMP-9 secretion (42).

The results we have obtained point to MMP-10 as a player in gastric cell invasion mediated by *H. pylori.* Indeed, MMPs play an important role in the degradation of extracellular matrix, which is an important component of cellular invasion. We have previously shown that *H. pylori* T4SS-competent and CagA-positive strains induce AGS cell invasion *via* c-Met receptor activation and increased activities of MMP-2 and MMP-9 (49). c-Met has a well-documented participation in cell invasion (3, 18, 25). It is plausible that phosphorylation of tyrosine residues at the c-Met receptor intracellular domains mediated by CagA results in the phosphorylation and binding of adaptor proteins and activation of signal transducers, including Nck and Src, eventually leading to MMP-10 expression and cell invasion.

We have previously observed that the increase in the proteolytic activities of MMP-2 and MMP-9 upon *H. pylori* infection, significantly decreased after silencing c-Met expression, suggesting that c-Met is involved in the activation of these MMPs (49). Adding to this observation, it has been shown that MMP-10 cleaves and activates pro-MMP-9 (41). Whether c-Met-mediated MMP-10 expression plays a role in pro-MMP-9 activation in the context of *H. pylori* infection will be an interesting topic of further studies.

In conclusion, our results show that MMP-10 expression is induced in gastric epithelial cells by CagA-positive *H. pylori* strains *via* the c-Met and EGF receptors, in a process that involves Nck and Src, and also the ERK and JNK pathways. Our results also demonstrate that MMP-10 is involved in *H. pylori*-mediated gastric cell invasion. In the future, it will be interesting to address whether targeting MMPs in the context of *H. pylori* infection, either by direct inhibitors or by targeting the signaling pathways that up-regulate MMP expression, will improve the outcome of the infection.

FIGURE LEGENDS

Figure 1. ***H. pylori increases MMP-10 mRNA expression, protein secretion, and activity.*** AGS cells were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. **(A)** MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells **(B)** Secreted MMP-10 was analyzed in the conditioned medium by western-blot. **(C)** MMP-10 activity in conditioned medium was analyzed by FRET. Data correspond to the mean values ± standard errors and are representative of, at least, three independent experiments. *, significantly different from uninfected cells.

Figure 2. ***H. pylori-mediated MMP-10 expression is CagA dependent.*** AGS cells were infected with *H. pylori* for 24 hours at a MOI of 100 and MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells. **(A)** Infection with *H. pylori* wild-type strain 84183 and its *cagA* (84183*ΔcagA*) and *cagE* (84183*ΔcagE*) mutants. **(B)** Infection with a panel of *H. pylori* isolates with known *cagA* status. Data correspond to the mean values ± standard errors and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells infected with wild-type *H. pylori* 84183.

Figure 3. ***H. pylori increases MMP-10 via c-Met and EGFR.*** AGS cells were treated with HGF at 150 or 250 ng/mL **(A)** and EGF at 50 or 150 ng/mL **(B)** for 24 hours. **(C-D)** AGS cells were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100, after the transient tranfection with a Non-silencing siRNA (NS) or an siRNA directed to c-Met (si c-Met) **(C)**, or after treatment with the EGFR chemical inhibitor AG-1478 at a final concentration of 5µM (iEGFR), or with dimethyl sulfoxide vehicle (DMSO), 1 hour before the infection **(D)**. **(A-D)** MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells **(A-B)**, cells treated with a Non-silencing siRNA **(C)** or with DMSO **(D)**. Data correspond to the mean values ± standard errors and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells infected with *H. pylori* 26695.

Figure 4. *c-Src and Nck, but not PLC-γ, are involved in H. pylori-induced MMP-10 expression.* **(A-C)** AGS cells alone or transiently transfected with a Non-silencing siRNA (NS) or with siRNAs directed to Nck, PLC-γ and Src, or **(D)** after a treatment for 1 hour with the Src family kinases chemical inhibitor PP2 (iSrc), or DMSO, were infected with *H. pylori* strain 26695 for 24 hours at a MOI of 100. MMP-10 expression was analyzed by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to cells treated with a Non-silencing siRNA (A-C) or with DMSO (D). Data correspond to the mean values ± standard errors and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from non-silenced cells or cells trated with DMSO.

Figure 5. *H. pylori increases MMP-10 expression through ERK and JNK, but not p38 pathways.* AGS cells were treated 1 hour before the infection with dimethyl sulfoxide vehicle (DMSO) or with chemical inhibitors of ERK1/2 (U-0126, 25 µM; iERK) **(A)**, JNK (SP600125, 20 µM; iJNK) **(A)**, and p38 (SB203580, 20 µM; ip38) **(B)**. **(A-B)** After treatment AGS cells were infected with *H. pylori* 26695 for 24 hours at a MOI of 100. MMP-10 expression was evaluated by qRT-PCR. MMP-10 expression levels were normalized to GAPDH expression and results are presented as fold differences relative to uninfected cells treated with DMSO. Data correspond to the mean values ± standard errors and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells infected with *H. pylori* 26695.

Figure 6. MMP-10 is involved in *H. pylori*-mediated cell invasion. (A) AGS cells were treated with a Non-silencing siRNA or a siRNA directed to MMP-10, and co-cultured with *H. pylori* for 48 hours. The efficiency of the knockdown was evaluated by qRT-PCR. **(B)** AGS cells treated with a Non-silencing siRNA or an siRNA directed to MMP-10, were co-cultured with *H. pylori* for 24 hours at a MOI of 100 on Matrigel coated filters. Data correspond to the mean values ± standard errors and are representative of three independent experiments. *, significantly different from uninfected cells; **, significantly different from cells infected with *H. pylori* 60190.

References

1. **Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow.** 2003. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science **300:**1430-4.
2. **Aung, P. P., N. Oue, Y. Mitani, H. Nakayama, K. Yoshida, T. Noguchi, A. K. Bosserhoff, and W. Yasui.** 2006. Systematic search for gastric cancer-specific genes based on SAGE data: melanoma inhibitory activity and matrix metalloproteinase-10 are novel prognostic factors in patients with gastric cancer. Oncogene **25:**2546-57.
3. **Bardelli, A., P. Longati, D. Gramaglia, C. Basilico, L. Tamagnone, S. Giordano, D. Ballinari, P. Michieli, and P. M. Comoglio.** 1998. Uncoupling signal transducers from oncogenic MET mutants abrogates cell transformation and inhibits invasive growth. Proc Natl Acad Sci U S A **95:**14379-83.
4. **Bebb, J. R., D. P. Letley, R. J. Thomas, F. Aviles, H. M. Collins, S. A. Watson, N. M. Hand, A. Zaitoun, and J. C. Atherton.** 2003. Helicobacter pylori upregulates matrilysin (MMP-7) in epithelial cells in vivo and in vitro in a Cag dependent manner. Gut **52:**1408-13.
5. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with Helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **55:**2111-5.
6. **Bodey, B., B. Bodey, Jr., S. E. Siegel, and H. E. Kaiser.** 2000. Immunocytochemical detection of MMP-3 and -10 expression in hepatocellular carcinomas. Anticancer Res **20:**4585-90.
7. **Brusevold, I. J., M. Aasrum, M. Bryne, and T. Christoffersen.** 2012. Migration induced by epidermal and hepatocyte growth factors in oral squamous carcinoma cells in vitro: role of MEK/ERK, p38 and PI-3 kinase/Akt. J Oral Pathol Med.
8. **Chakraborti, S., M. Mandal, S. Das, A. Mandal, and T. Chakraborti.** 2003. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem **253:**269-85.
9. **Chang, J. H., S. Gill, J. Settleman, and S. J. Parsons.** 1995. c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. J Cell Biol **130:**355-68.
10. **Chiang, G. J., B. R. Billmeyer, D. Canes, J. Stoffel, A. Moinzadeh, C. A. Austin, M. Kosakowski, K. M. Rieger-Christ, J. A. Libertino, and I. C. Summerhayes.** 2005. The src-family kinase inhibitor PP2 suppresses the in vitro invasive phenotype of bladder carcinoma cells via modulation of Akt. BJU Int **96:**416-22.
11. **Churin, Y., L. Al-Ghoul, O. Kepp, T. F. Meyer, W. Birchmeier, and M. Naumann.** 2003. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol **161:**249-55.
12. **Churin, Y., E. Kardalinou, T. F. Meyer, and M. Naumann.** 2001. Pathogenicity island-dependent activation of Rho GTPases Rac1 and Cdc42 in Helicobacter pylori infection. Mol Microbiol **40:**815-23.
13. **Crabtree, J. E., J. D. Taylor, J. I. Wyatt, R. V. Heatley, T. M. Shallcross, D. S. Tompkins, and B. J. Rathbone.** 1991. Mucosal IgA recognition of Helicobacter pylori 120 kDa protein, peptic ulceration, and gastric pathology. Lancet **338:**332-5.
14. **Crawford, H. C., U. S. Krishna, D. A. Israel, L. M. Matrisian, M. K. Washington, and R. M. Peek, Jr.** 2003. Helicobacter pylori strain-selective induction of matrix metalloproteinase-7 in vitro and within gastric mucosa. Gastroenterology **125:**1125-36.
15. **Deraz, E. M., Y. Kudo, M. Yoshida, M. Obayashi, T. Tsunematsu, H. Tani, S. B. Siriwardena, M. R. Keikhaee, G. Qi, S. Iizuka, I. Ogawa, G. Campisi, L. Lo Muzio, Y. Abiko, A. Kikuchi, and T. Takata.** 2011. MMP-10/stromelysin-2 promotes invasion of head and neck cancer. PLoS One **6:**e25438.

16. **Egeblad, M., and Z. Werb.** 2002. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer **2:**161-74.
17. **Figueiredo, C., J. C. Machado, P. Pharoah, R. Seruca, S. Sousa, R. Carvalho, A. F. Capelinha, W. Quint, C. Caldas, L. J. van Doorn, F. Carneiro, and M. Sobrinho-Simoes.** 2002. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst **94:**1680-7.
18. **Furge, K. A., Y. W. Zhang, and G. F. Vande Woude.** 2000. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. Oncogene **19:**5582-9.
19. **Giannakis, M., S. L. Chen, S. M. Karam, L. Engstrand, and J. I. Gordon.** 2008. Helicobacter pylori evolution during progression from chronic atrophic gastritis to gastric cancer and its impact on gastric stem cells. Proc Natl Acad Sci U S A **105:**4358-63.
20. **Gill, J. H., I. G. Kirwan, J. M. Seargent, S. W. Martin, S. Tijani, V. A. Anikin, A. J. Mearns, M. C. Bibby, A. Anthoney, and P. M. Loadman.** 2004. MMP-10 is overexpressed, proteolytically active, and a potential target for therapeutic intervention in human lung carcinomas. Neoplasia **6:**777-85.
21. **Gonzalez, C. A., C. Figueiredo, C. B. Lic, R. M. Ferreira, M. L. Pardo, J. M. Ruiz Liso, P. Alonso, N. Sala, G. Capella, and J. M. Sanz-Anquela.** 2011. Helicobacter pylori cagA and vacA genotypes as predictors of progression of gastric preneoplastic lesions: a long-term follow-up in a high-risk area in Spain. Am J Gastroenterol **106:**867-74.
22. **Gooz, M., M. Shaker, P. Gooz, and A. J. Smolka.** 2003. Interleukin 1beta induces gastric epithelial cell matrix metalloproteinase secretion and activation during Helicobacter pylori infection. Gut **52:**1250-6.
23. **Gruenheid, S., R. DeVinney, F. Bladt, D. Goosney, S. Gelkop, G. D. Gish, T. Pawson, and B. B. Finlay.** 2001. Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. Nat Cell Biol **3:**856-9.
24. **Hake, M. J., K. Choowongkomon, O. Kostenko, C. R. Carlin, and F. D. Sonnichsen.** 2008. Specificity determinants of a novel Nck interaction with the juxtamembrane domain of the epidermal growth factor receptor. Biochemistry **47:**3096-108.
25. **Hasegawa, Y., M. Yamamoto, S. Maeda, and Y. Saitoh.** 1995. Hepatocyte growth-factor and its receptor C-met regulate both cell-growth and invasion of human pancreatic-cancer. Int J Oncol **7:**877-81.
26. **Herynk, M. H., J. Zhang, N. U. Parikh, and G. E. Gallick.** 2007. Activation of Src by c-Met overexpression mediates metastatic properties of colorectal carcinoma cells. J Exp Ther Oncol **6:**205-17.
27. **Jo, M., D. B. Stolz, J. E. Esplen, K. Dorko, G. K. Michalopoulos, and S. C. Strom.** 2000. Cross-talk between epidermal growth factor receptor and c-Met signal pathways in transformed cells. J Biol Chem **275:**8806-11.
28. **Kassis, J., J. Moellinger, H. Lo, N. M. Greenberg, H. G. Kim, and A. Wells.** 1999. A role for phospholipase C-gamma-mediated signaling in tumor cell invasion. Clin Cancer Res **5:**2251-60.
29. **Keates, S., Y. S. Hitti, M. Upton, and C. P. Kelly.** 1997. Helicobacter pylori infection activates NF-kappa B in gastric epithelial cells. Gastroenterology **113:**1099-109.
30. **Keates, S., A. C. Keates, S. Nath, R. M. Peek, Jr., and C. P. Kelly.** 2005. Transactivation of the epidermal growth factor receptor by cag+ Helicobacter pylori induces upregulation of the early growth response gene Egr-1 in gastric epithelial cells. Gut **54:**1363-9.
31. **Keates, S., S. Sougioultzis, A. C. Keates, D. Zhao, R. M. Peek, Jr., L. M. Shaw, and C. P. Kelly.** 2001. cag+ Helicobacter pylori induce transactivation of the epidermal growth factor receptor in AGS gastric epithelial cells. J Biol Chem **276:**48127-34.
32. **Kochhar, K. S., and A. P. Iyer.** 1996. Hepatocyte growth factor induces activation of Nck and phospholipase C-gamma in lung carcinoma cells. Cancer Lett **104:**163-9.

33. **Krueger, S., T. Hundertmark, T. Kalinski, U. Peitz, T. Wex, P. Malfertheiner, M. Naumann, and A. Roessner.** 2006. Helicobacter pylori encoding the pathogenicity island activates matrix metalloproteinase 1 in gastric epithelial cells via JNK and ERK. J Biol Chem **281:**2868-75.
34. **Lawler, K., G. O'Sullivan, A. Long, and D. Kenny.** 2009. Shear stress induces internalization of E-cadherin and invasiveness in metastatic oesophageal cancer cells by a Src-dependent pathway. Cancer Sci **100:**1082-7.
35. **Le, N. T., M. Xue, L. A. Castelnoble, and C. J. Jackson.** 2007. The dual personalities of matrix metalloproteinases in inflammation. Front Biosci **12:**1475-87.
36. **Lee, J., W. K. Kang, J. O. Park, S. H. Park, Y. S. Park, H. Y. Lim, J. Kim, J. Kong, M. G. Choi, T. S. Sohn, J. H. Noh, J. M. Bae, S. Kim, H. Lim do, K. M. Kim, and C. K. Park.** 2009. Expression of activated signal transducer and activator of transcription 3 predicts poor clinical outcome in gastric adenocarcinoma. APMIS **117:**598-606.
37. **Lin, M. T., B. R. Lin, C. C. Chang, C. Y. Chu, H. J. Su, S. T. Chen, Y. M. Jeng, and M. L. Kuo.** 2007. IL-6 induces AGS gastric cancer cell invasion via activation of the c-Src/RhoA/ROCK signaling pathway. Int J Cancer **120:**2600-8.
38. **Mathew, R., R. Khanna, R. Kumar, M. Mathur, N. K. Shukla, and R. Ralhan.** 2002. Stromelysin-2 overexpression in human esophageal squamous cell carcinoma: potential clinical implications. Cancer Detect Prev **26:**222-8.
39. **McCawley, L. J., and L. M. Matrisian.** 2001. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol **13:**534-40.
40. **Mueller, K. L., L. A. Hunter, S. P. Ethier, and J. L. Boerner.** 2008. Met and c-Src cooperate to compensate for loss of epidermal growth factor receptor kinase activity in breast cancer cells. Cancer Res **68:**3314-22.
41. **Nakamura, H., Y. Fujii, E. Ohuchi, E. Yamamoto, and Y. Okada.** 1998. Activation of the precursor of human stromelysin 2 and its interactions with other matrix metalloproteinases. Eur J Biochem **253:**67-75.
42. **Nam, Y. H., E. Ryu, D. Lee, H. J. Shim, Y. C. Lee, and S. T. Lee.** 2011. CagA phosphorylation-dependent MMP-9 expression in gastric epithelial cells. Helicobacter **16:**276-83.
43. **Nath, D., N. J. Williamson, R. Jarvis, and G. Murphy.** 2001. Shedding of c-Met is regulated by crosstalk between a G-protein coupled receptor and the EGF receptor and is mediated by a TIMP-3 sensitive metalloproteinase. J Cell Sci **114:**1213-20.
44. **Naumann, M., and J. E. Crabtree.** 2004. Helicobacter pylori-induced epithelial cell signalling in gastric carcinogenesis. Trends Microbiol **12:**29-36.
45. **Naumann, M., S. Wessler, C. Bartsch, B. Wieland, A. Covacci, R. Haas, and T. F. Meyer.** 1999. Activation of activator protein 1 and stress response kinases in epithelial cells colonized by Helicobacter pylori encoding the cag pathogenicity island. J Biol Chem **274:**31655-62.
46. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science **287:**1497-500.
47. **Ogata, T., H. Shibamura, G. Tromp, M. Sinha, K. A. Goddard, N. Sakalihasan, R. Limet, G. L. MacKean, C. Arthur, T. Sueda, S. Land, and H. Kuivaniemi.** 2005. Genetic analysis of polymorphisms in biologically relevant candidate genes in patients with abdominal aortic aneurysms. J Vasc Surg **41:**1036-42.
48. **Okano, Y., K. Mizuno, S. Osada, T. Nakamura, and Y. Nozawa.** 1993. Tyrosine phosphorylation of phospholipase C gamma in c-met/HGF receptor-stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. Biochem Biophys Res Commun **190:**842-8.
49. **Oliveira, M. J., A. C. Costa, A. M. Costa, L. Henriques, G. Suriano, J. C. Atherton, J. C. Machado, F. Carneiro, R. Seruca, M. Mareel, A. Leroy, and C. Figueiredo.** 2006.

Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. J Biol Chem **281:**34888-96.

50. **Oliveira, M. J., A. M. Costa, A. C. Costa, R. M. Ferreira, P. Sampaio, J. C. Machado, R. Seruca, M. Mareel, and C. Figueiredo.** 2009. CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses Helicobacter pylori-induced cell-invasive phenotype. J Infect Dis **200:**745-55.
51. **Oser, M., A. Dovas, D. Cox, and J. Condeelis.** Nck1 and Grb2 localization patterns can distinguish invadopodia from podosomes. Eur J Cell Biol **90:**181-8.
52. **P, O. C., P. H. Rhys-Evans, and S. A. Eccles.** 2001. Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg **127:**813-20.
53. **Parks, W. C., C. L. Wilson, and Y. S. Lopez-Boado.** 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol **4:**617-29.
54. **Peek, R. M., Jr., and M. J. Blaser.** 2002. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer **2:**28-37.
55. **Peek, R. M., Jr., G. G. Miller, K. T. Tham, G. I. Perez-Perez, X. Zhao, J. C. Atherton, and M. J. Blaser.** 1995. Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. Lab Invest **73:**760-70.
56. **Pillinger, M. H., N. Marjanovic, S. Y. Kim, Y. C. Lee, J. U. Scher, J. Roper, A. M. Abeles, P. I. Izmirly, M. Axelrod, M. Y. Pillinger, S. Tolani, V. Dinsell, S. B. Abramson, and M. J. Blaser.** 2007. Helicobacter pylori stimulates gastric epithelial cell MMP-1 secretion via CagA-dependent and -independent ERK activation. J Biol Chem **282:**18722-31.
57. **Pillinger, M. H., N. Marjanovic, S. Y. Kim, J. U. Scher, P. Izmirly, S. Tolani, V. Dinsell, Y. C. Lee, M. J. Blaser, and S. B. Abramson.** 2005. Matrix metalloproteinase secretion by gastric epithelial cells is regulated by E prostaglandins and MAPKs. J Biol Chem **280:**9973-9.
58. **Rahimi, N., W. Hung, E. Tremblay, R. Saulnier, and B. Elliott.** 1998. c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. J Biol Chem **273:**33714-21.
59. **Reunanen, N., J. Westermarck, L. Hakkinen, T. H. Holmstrom, I. Elo, J. E. Eriksson, and V. M. Kahari.** 1998. Enhancement of fibroblast collagenase (matrix metalloproteinase-1) gene expression by ceramide is mediated by extracellular signal-regulated and stress-activated protein kinase pathways. J Biol Chem **273:**5137-45.
60. **Ridley, S. H., S. J. Sarsfield, J. C. Lee, H. F. Bigg, T. E. Cawston, D. J. Taylor, D. L. DeWitt, and J. Saklatvala.** 1997. Actions of IL-1 are selectively controlled by p38 mitogen-activated protein kinase: regulation of prostaglandin H synthase-2, metalloproteinases, and IL-6 at different levels. J Immunol **158:**3165-73.
61. **Rivera, G. M., S. Antoku, S. Gelkop, N. Y. Shin, S. K. Hanks, T. Pawson, and B. J. Mayer.** 2006. Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. Proc Natl Acad Sci U S A **103:**9536-41.
62. **Saghizadeh, M., D. J. Brown, R. Castellon, M. Chwa, G. H. Huang, J. Y. Ljubimova, S. Rosenberg, K. S. Spirin, R. B. Stolitenko, W. Adachi, S. Kinoshita, G. Murphy, L. J. Windsor, M. C. Kenney, and A. V. Ljubimov.** 2001. Overexpression of matrix metalloproteinase-10 and matrix metalloproteinase-3 in human diabetic corneas: a possible mechanism of basement membrane and integrin alterations. Am J Pathol **158:**723-34.
63. **Selbach, M., S. Moese, C. R. Hauck, T. F. Meyer, and S. Backert.** 2002. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. J Biol Chem **277:**6775-8.
64. **Sokolova, O., M. Vieth, and M. Naumann.** 2012. Protein kinase C isozymes regulate matrix metalloproteinase-1 expression and cell invasion in Helicobacter pylori infection. Gut.

65. **Stamenkovic, I.** 2003. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol **200:**448-64.
66. **Stylli, S. S., T. T. Stacey, A. M. Verhagen, S. S. Xu, I. Pass, S. A. Courtneidge, and P. Lock.** 2009. Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. J Cell Sci **122:**2727-40.
67. **Suerbaum, S., and P. Michetti.** 2002. Helicobacter pylori infection. N Engl J Med **347:**1175-86.
68. **Takaishi, S., and T. C. Wang.** 2007. Gene expression profiling in a mouse model of Helicobacter-induced gastric cancer. Cancer Sci **98:**284-93.
69. **Tammer, I., S. Brandt, R. Hartig, W. Konig, and S. Backert.** 2007. Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. Gastroenterology **132:**1309-19.
70. **Terakado, M., Y. Gon, A. Sekiyama, I. Takeshita, Y. Kozu, K. Matsumoto, N. Takahashi, and S. Hashimoto.** 2011. The Rac1/JNK pathway is critical for EGFR-dependent barrier formation in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol **300:**L56-63.
71. **Thorns, V., G. F. Walter, and C. Thorns.** 2003. Expression of MMP-2, MMP-7, MMP-9, MMP-10 and MMP-11 in human astrocytic and oligodendroglial gliomas. Anticancer Res **23:**3937-44.
72. **Tsai, S. Y., Y. L. Huang, W. H. Yang, and C. H. Tang.** 2012. Hepatocyte growth factor-induced BMP-2 expression is mediated by c-Met receptor, FAK, JNK, Runx2, and p300 pathways in human osteoblasts. Int Immunopharmacol.
73. **Vaalamo, M., M. L. Karjalainen-Lindsberg, P. Puolakkainen, J. Kere, and U. Saarialho-Kere.** 1998. Distinct expression profiles of stromelysin-2 (MMP-10), collagenase-3 (MMP-13), macrophage metalloelastase (MMP-12), and tissue inhibitor of metalloproteinases-3 (TIMP-3) in intestinal ulcerations. Am J Pathol **152:**1005-14.
74. **van Doorn, L. J., C. Figueiredo, R. Rossau, G. Jannes, M. van Asbroek, J. C. Sousa, F. Carneiro, and W. G. Quint.** 1998. Typing of Helicobacter pylori vacA gene and detection of cagA gene by PCR and reverse hybridization. J Clin Microbiol **36:**1271-6.
75. **Wallasch, C., J. E. Crabtree, D. Bevec, P. A. Robinson, H. Wagner, and A. Ullrich.** 2002. Helicobacter pylori-stimulated EGF receptor transactivation requires metalloprotease cleavage of HB-EGF. Biochem Biophys Res Commun **295:**695-701.
76. **Wessler, S., M. Hocker, W. Fischer, T. C. Wang, S. Rosewicz, R. Haas, B. Wiedenmann, T. F. Meyer, and M. Naumann.** 2000. Helicobacter pylori activates the histidine decarboxylase promoter through a mitogen-activated protein kinase pathway independent of pathogenicity island-encoded virulence factors. J Biol Chem **275:**3629-36.
77. **Wilkins-Port, C. E., C. E. Higgins, J. Freytag, S. P. Higgins, J. A. Carlson, and P. J. Higgins.** 2007. PAI-1 is a Critical Upstream Regulator of the TGF-beta1/EGF-Induced Invasive Phenotype in Mutant p53 Human Cutaneous Squamous Cell Carcinoma. J Biomed Biotechnol **2007:**85208.
78. **Wilkins-Port, C. E., and P. J. Higgins.** 2007. Regulation of extracellular matrix remodeling following transforming growth factor-beta1/epidermal growth factor-stimulated epithelial-mesenchymal transition in human premalignant keratinocytes. Cells Tissues Organs **185:**116-22.
79. **Wroblewski, L. E., P. J. Noble, A. Pagliocca, D. M. Pritchard, C. A. Hart, F. Campbell, A. R. Dodson, G. J. Dockray, and A. Varro.** 2003. Stimulation of MMP-7 (matrilysin) by Helicobacter pylori in human gastric epithelial cells: role in epithelial cell migration. J Cell Sci **116:**3017-26.
80. **Xu, K. P., and F. S. Yu.** 2007. Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. Invest Ophthalmol Vis Sci **48:**2242-8.

81. **Yin, Y., A. M. Grabowska, P. A. Clarke, E. Whelband, K. Robinson, R. H. Argent, A. Tobias, R. Kumari, J. C. Atherton, and S. A. Watson.** 2010. Helicobacter pylori potentiates epithelial:mesenchymal transition in gastric cancer: links to soluble HB-EGF, gastrin and matrix metalloproteinase-7. Gut **59:**1037-45.

Figure 1

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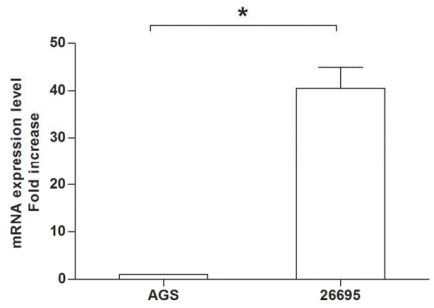
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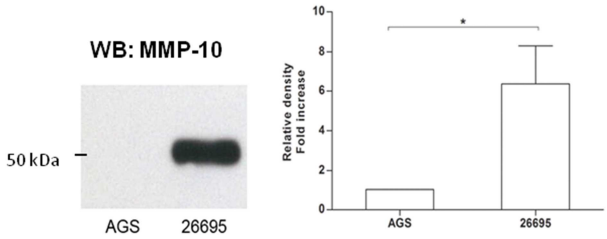
B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C

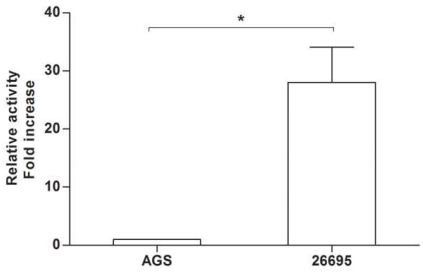
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Figure 2

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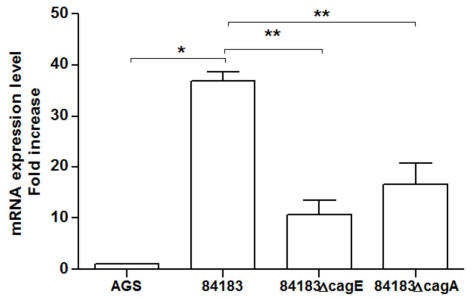
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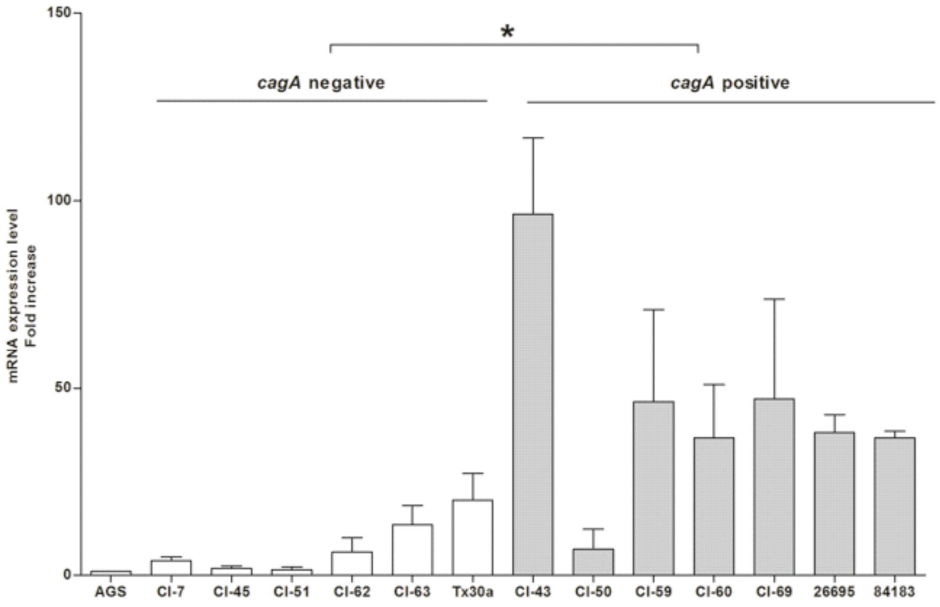
B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84183

Figure 3

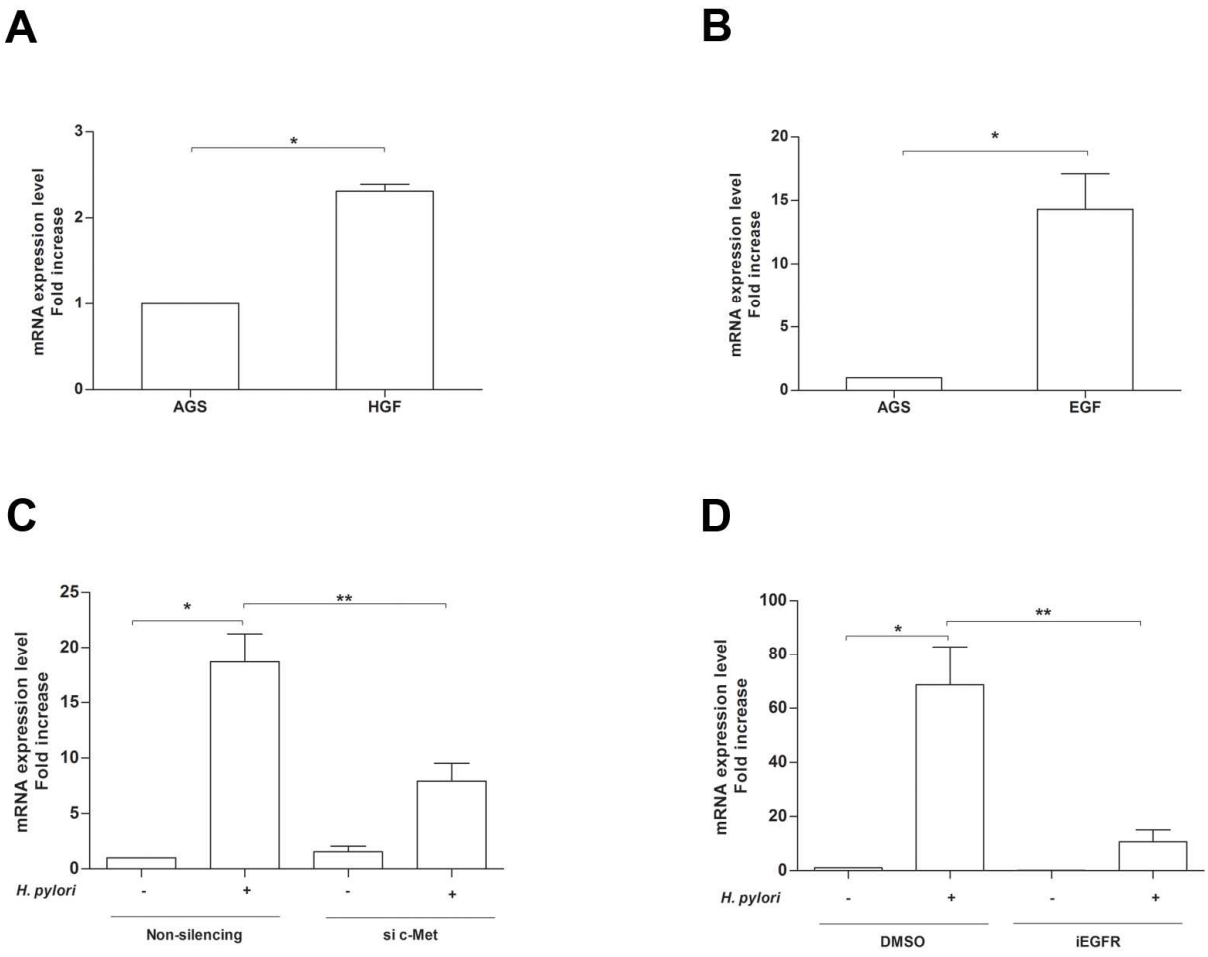
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Figure 4

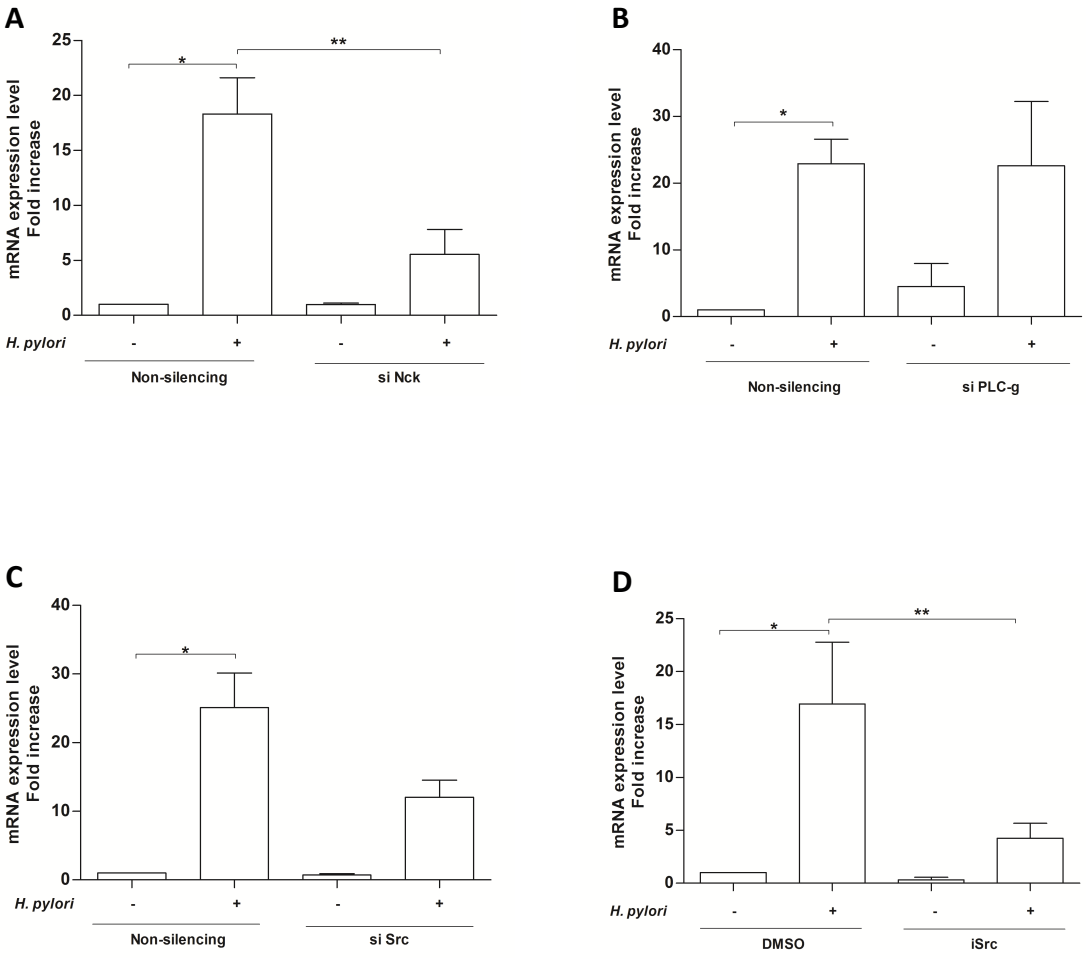
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Figure 5

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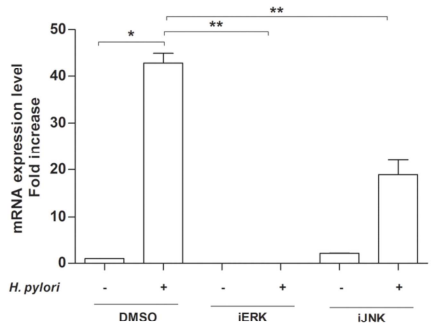
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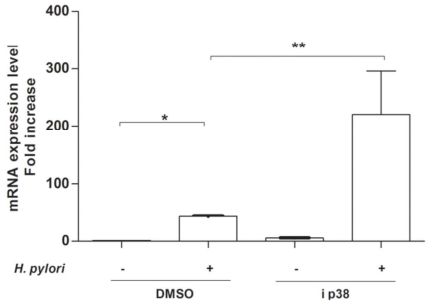
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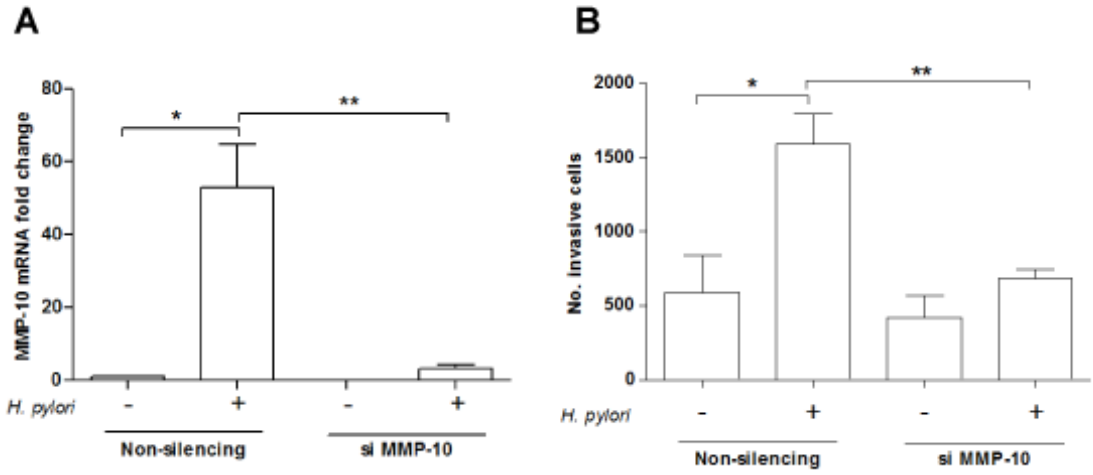
Figure 6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Non-silencing
si MMP-10
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No. invasive cells
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H. pylori
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Non-silencing
si MMP-10

Paper II

CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses Helicobacter pylori-induced cell-invasive phenotype

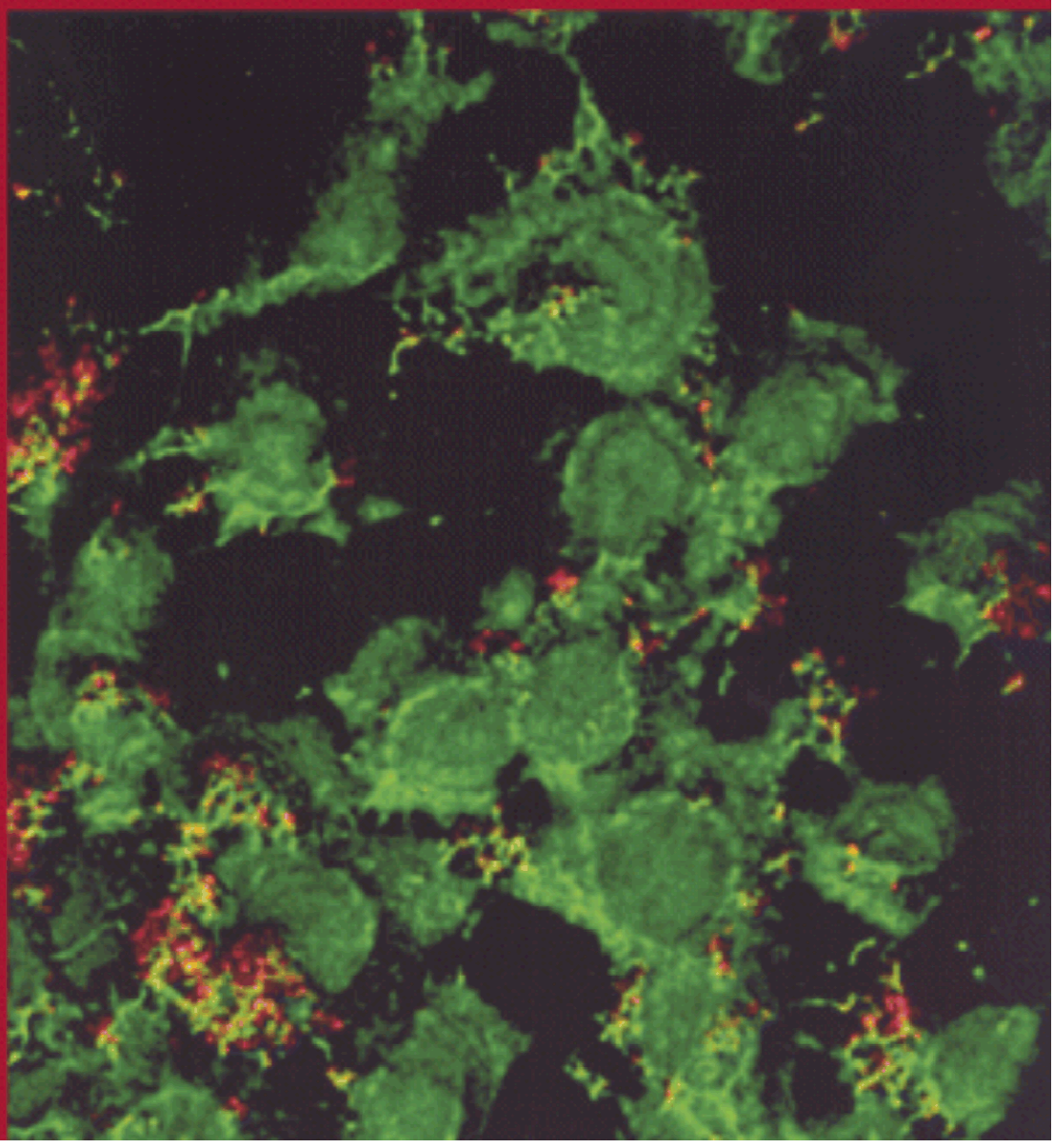
Costa AM*, Oliveira MJ*, Costa AC, Ferreira RM, Sampaio P, Machado JC, Seruca R, Mareel M, Figueiredo C.

* shared first co-authorship

Journal of Infectious Diseases 2009 Sep 1; 200(5):745-55.

1 September 2009
Volume 200
Number 5

The Journal of Infectious Diseases



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On the cover: Image of E-cadherin-transduced AGS gastric cells showing colocalization (*yellow)* between p120-catenin *(green)* and *Helicobacter pylori* CagA *(red)* 1 hour after infection

MAJOR ARTICLE

CagA Associates with c-Met, E-Cadherin, and p120-Catenin in a Multiproteic Complex That Suppresses *Helicobacter pylori*–Induced Cell-Invasive Phenotype

Maria Jose Oliveira,[1,a] Angela Margarida Costa,[1,2,a] Ana Catarina Costa,[1,2] Rui Manuel Ferreira,[1] Paula Sampaio,[3] Jose Carlos Machado,[1,2] Raquel Seruca,[1,2] Marc Mareel,[4] and Ceu Figueiredo[1,2]

[1]Institute of Molecular Pathology and Immunology of the University of Porto, [2]Department of Pathology, Medical Faculty of Porto, and [3]Institute of Molecular and Cellular Biology, Porto, Portugal; and [4]Laboratory of Experimental Cancer, Ghent University Hospital, Ghent, Belgium

Background. *Helicobacter pylori* induces an invasive phenotype in gastric epithelial cells through a mechanism that requires the type IV secretion system and the phosphorylation of c-Met. The E-cadherin–catenin complex is a major component of the adherens junctions and functions as an invasion suppressor. We investigated whether E-cadherin has a role in *H. pylori*–induced, c-Met phosphorylation-dependent cell-invasive phenotype.

Methods. AGS cells that lack E-cadherin and that are invasive to *H. pylori* stimulation were transduced with E-cadherin and infected with *H. pylori*. NCI-N87 cells, which endogenously express E-cadherin, were also used for infection experiments.

Results. E-cadherin was sufficient to suppress not only *H. pylori*–mediated cell-invasive phenotype but also c-Met and p120-catenin tyrosine phosphorylation. *H. pylori* infection led to increased interactions between E-cadherin and p120-catenin, c-Met and E-cadherin, and c-Met and p120-catenin. Using in vitro infection assays, we showed that *H. pylori* CagA interacts with E-cadherin, p120-catenin, and c-Met. Finally, using small interfering RNA, we showed that interactions between CagA and E-cadherin and between CagA and p120-catenin were established through c-Met.

Conclusions. We suggest that *H. pylori* alters the E-cadherin–catenin complex, leading to formation of a multiproteic complex composed of CagA, c-Met, E-cadherin, and p120-catenin. This complex abrogates c-Met and p120-catenin tyrosine phosphorylation and suppresses the cell-invasive phenotype induced by *H. pylori*.

Helicobacter pylori colonizes the stomach of more than one-half of the human population and is a risk factor for gastric carcinoma development [1–3]. In the past 2 decades, many studies have been performed in an attempt to identify *H. pylori* virulence factors, to evaluate their biological effect, and to clarify their contribution to infection and disease [4–6].

The *cag* pathogenicity island (*cag*PAI) is one of the best characterized *H. pylori* virulence factors. Studies have demonstrated that individuals infected with *H. pylori cag*PAI–positive strains present increased inflammation and have a higher risk of developing peptic ulcer disease and gastric carcinoma, compared with individuals infected with *cag*PAI-negative strains [3, 7]. The *cag*PAI is a cluster of ~30 genes that encode components of a type IV secretion system (T4SS). Once attached to the host cell, *cag*PAI-positive strains inject CagA into the host cytoplasm via the T4SS [8]. Inside the host cell, CagA localizes to the inner surface of the plasma membrane [9] and targets cell-cell junctions, namely the tight and the adherens junctions [10, 11]. CagA is also described to target the tyrosine kinase c-Met receptor, leading to its activation and induction of a motogenic phenotype [12].

The E-cadherin–catenin complex is a major component of adherens junctions, functioning as a cell-cell adhesion complex, a regulator of signaling pathways,

Received 12 July 2008; accepted 4 December 2008; electronically published 15 July 2009.

Potential conflicts of interest: none reported.

Financial support: Fundação para a Ciência e a Tecnologia (POCI/SAU-IMI/56681/2004, ERA-NET Pathogenomics ERA-PTG/0002/2006, and REEQ/218/SAU/2005) and Fundação Calouste Gulbenkian (Proj. 76448).

Presented in part: Digestive Diseases Week 2008, San Diego, CA, May 2008 (abstract 26).

[a] M.J.O. and A.M.C. contributed equally to this article.

Reprints or correspondence: Dr. Ceu Figueiredo, IPATIMUP, Rua Dr. Roberto Frias, S/N 4200-465 Porto, Portugal (cfigueiredo@ipatimup.pt).

The Journal of Infectious Diseases 2009; 200:745–55

0022-1899/2009/20005-0013$15.00
DOI: 10.1086/604727

and a bacterial cell receptor [13, 14]. E-cadherin molecules establish homophilic interactions through their extracellular domains to form adhesive complexes. The extracellular domain of E-cadherin also participates in heterophilic interactions with other proteins, such as the epidermal growth factor receptor [15, 16], the c-Met receptor [17, 18], and the internalin A surface receptor of *Listeria monocytogenes* [13]. The cytoplasmic domain of E-cadherin interacts directly with β- and p120-catenins via separate interaction domains, and these molecules physically interact or regulate the actin cytoskeleton [19]. E-cadherin loss of function by mutation or promoter hypermethylation is a common feature of many cancers, including diffuse-type gastric carcinomas [20, 21]. *H. pylori* infection has been associated with E-cadherin gene promoter methylation [22, 23] and affects the functionality of the E-cadherin–catenin complex [11, 24].

We previously reported that *H. pylori* strains containing a functional T4SS induce an invasive phenotype of gastric cells through a c-Met–receptor dependent signaling pathway [25]. In the present study, we revealed that E-cadherin is sufficient to suppress *H. pylori*–induced cell-invasive phenotype. Our in vitro infection assays confirm that CagA interacts with E-cadherin, and for the first time to our knowledge, we revealed that CagA also interacts with p120-catenin. Furthermore, we revealed that CagA, c-Met, E-cadherin, and p120-catenin form in a multiproteic complex.

MATERIALS AND METHODS

Cell culture and complementary DNA (cDNA) transduction. AGS, NCI-N87 (American Type Culture Collection [ATCC]), and IPA220 cells derived from human gastric carcinomas were maintained in RPMI 1640 (Invitrogen) with 10% fetal bovine serum, 2.5 μg/mL fungizone (Bristol-Myers Squibb), 200 μg/mL streptomycin, and 200 IU/mL penicillin at 37°C under 5% carbon dioxide. Cells were stably transduced using a pLenti6/V5 construct containing E-cadherin cDNA, as described elsewhere [26]. For clonal selection, 5 μg/mL of blasticidin was added to the medium.

Immunocytochemistry and confocal microscopy. Confluent monolayers were grown on glass coverslips, fixed in ice-cold methanol, incubated in 5% bovine serum albumin (Sigma-Aldrich), and immunostained with mouse monoclonal anti–E-cadherin, anti–α-catenin, or anti–p120-catenin antibodies (BD Biosciences–Transduction Laboratories) or with rabbit polyclonal anti–β-catenin (Sigma-Aldrich) or anti-CagA (Santa Cruz Biotechnology) antibodies. Rabbit anti-mouse or swine anti-rabbit–fluorescein isothiocyanate conjugate (Amersham) and goat anti–mouse-Alexa488 or anti–rabbit-Alexa594 conjugate were used as secondary antibodies (Molecular Probes). A Leica DMRE2 fluorescence microscope or a SP2-SE-AOBS laser-scanning confocal microscope (Leica Microsystems) were used. Confocal images were deconvolved with Huygens Pro3.2 (SVI).

Aggregation assays. Cells were trypsinized, resuspended as single cells, and added to 96-well agar-coated plates. As control for E-cadherin–mediated aggregation, AGSEcad cells were incubated for 48 h with an anti–E-cadherin antibody (MB2). The ability of cells to aggregate or persist as a single-cell suspension was evaluated microscopically.

Bacterial strains and growth conditions. Bacteria were grown in tryptic soy agar supplemented with 5% sheep blood (BioMérieux) and were incubated for 48 h at 37°C under a microaerophilic atmosphere. Experiments were performed with *H. pylori* strain 26695 (ATCC 700392; *cag* PAI$^+$), 60190 (ATCC 49503; *cag* PAI$^+$), or an insertion mutant for *cagA* (60190 CagA$^-$).

Infection of gastric cells. Eighty percent confluent monolayers were washed in phosphate-buffered saline and were incubated in serum and antibiotic-free medium (Invitrogen). *H. pylori* was added at a multiplicity of infection of 100. Cultures were maintained at 37°C under a 5% carbon dioxide atmosphere.

Invasion assays. Twenty-four–well Matrigel-coated invasion inserts of 8 μm pore-size filters (BD Biosciences) were incubated for 1 h at 37°C with antibiotic-free medium. After rehydration, 5×10^4 cells were incubated for 24 h at 37°C on top of the filters, in the presence or absence of *H. pylori.* Filters were washed, fixed in 4% paraformaldehyde, and mounted in Vectashield with 4′-6-diamidino-2-phenylindole (Vector Laboratories). Invasive cells were scored in at least 25 microscopic fields (20× objective).

Preparation of cell lysates and immunoprecipitation. Cells were lysed in 20 mmol/L Tris-hydrochloride (pH, 7.5), 150 mmol/L sodium chloride, 1% Triton X-100, 1% NP-40, 3 mmol/L sodium vanadate, 20 mmol/L sodium fluoride, 1 mmol/L phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Seven hundred fifty micrograms of proteins were incubated overnight at 4°C with mouse monoclonal antiphosphotyrosine, anti–E-cadherin, anti–p120-catenin, or anti–c-Met antibodies. Immunoprecipitated complexes were incubated for 1 h with protein G-sepharose beads (Amersham), which were washed and pre-incubated in 1% bovine serum albumin solution. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Immunoblot analysis. Proteins were transferred onto Hybond nitrocellulose membranes (Amersham), blocked with 4% bovine serum albumin (phosphorylated proteins) or 5% nonfat milk in phosphate-buffered saline plus 0.5% Tween-20 (overall protein), and incubated for 1 h with mouse monoclonal anti–E-cadherin, anti–p120-catenin, anti-CagA, or rabbit polyclonal anti–c-Met antibodies (Santa Cruz Biotechnology). Goat anti-rabbit (Santa Cruz Biotechnology) or rabbit anti-mouse (Amer-

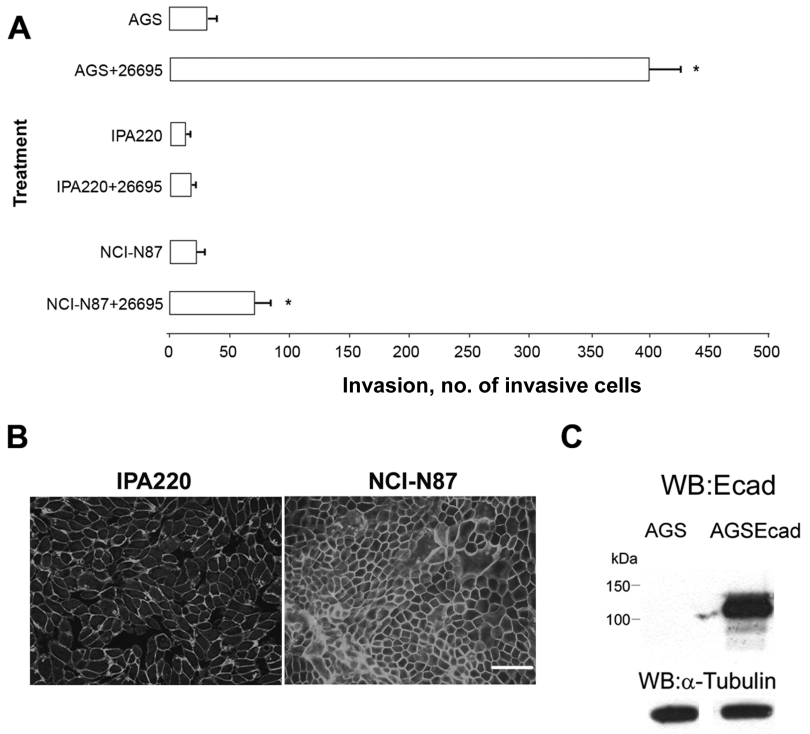


Figure 1. The E-cadherin–catenin complex suppresses *Helicobacter pylori*–mediated cell-invasive phenotype. *A,* Invasion assays of AGS, IPA220, and NCI-N87 cells infected or not infected for 24 h with *H. pylori* on Matrigel-coated filters. Graphics represent the mean value of invasion ± standard deviation and are representative of 3 independent experiments. *B,* E-cadherin staining of IPA220 and NCI-N87 cells. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole. Scale bar represents 100 μm. *C,* Western blot for E-cadherin of AGS cells and of AGS cells stably transduced with human E-cadherin (AGSEcad). α-Tubulin immunostaining was used as loading control. *Significantly different from uninfected cells.

sham) HRP-conjugated secondary antibodies were used; subsequently, ECL detection (Amersham) was performed.

Small interference RNA (siRNA) transfer. siRNA targeting c-Met, previously tested for knockdown efficiency, were from Qiagen. Nonsilencing siRNA duplexes were used as negative control. Fifty percent confluent monolayers were washed and incubated in serum- and antibiotic-free medium and were transiently transfected with 80 nmol/L or 100 nmol/L of siRNA for AGSEcad and NCI-N87, respectively, with use of Lipofectamine 2000 (Invitrogen).

Statistical analysis. Data were analyzed using Student's *t* test and were expressed as mean values of at least 3 independent experiments ± standard deviations. Differences were considered to be statistically significant at $P < .05$.

RESULTS

Cells expressing a functional E-cadherin–catenin complex are not responsive to* H. pylori*–induced cell invasion. We previously reported that *H. pylori* induces an invasive phenotype of AGS cells into extracellular matrix components through a mechanism that requires the T4SS and the phosphorylation of the c-Met receptor [25]. Because the E-cadherin–catenin complex functions as an invasion suppressor [27, 28], we investigated whether the complex has a role in *H. pylori*–mediated cell-invasive phenotype. For that, the human gastric carcinoma cells AGS, IPA220, and NCI-N87 were confronted with *H. pylori* on Matrigel invasion assays. We observed that, in contrast to AGS cells, which acquired an invasive phenotype in response to *H. pylori,* cell lines IPA220 and NCI-N87 were significantly less responsive to *H. pylori* (figure 1*A*). From the panel of gastric cell lines tested, IPA220 and NCI-N87 cells have an intact E-cadherin–catenin complex (figure 1*B*), whereas AGS cells harbor an E-cadherin mutation, leading to a truncated form of the protein that is not expressed (C.O., personal communication) (figures 1*C* and 2*A*). These results point to a role of the E-cadherin–catenin complex in the inhibition of *H. pylori*–mediated invasion.

AGS cells expressing E-cadherin assemble a functional E-cadherin–catenin complex. To investigate the role of E-cadherin in *H. pylori*–mediated invasive phenotype, we stably transduced AGS cells with E-cadherin. Before transduction, we confirmed by Western blot and immunocytochemistry that AGS cells do not express E-cadherin (figures 1*C* and 2*A*). AGS were stably transduced using a lentivirus transduction system with human E-cadherin cDNA. For clone selection, transduced cells were grown in medium with blasticidin, and each clone

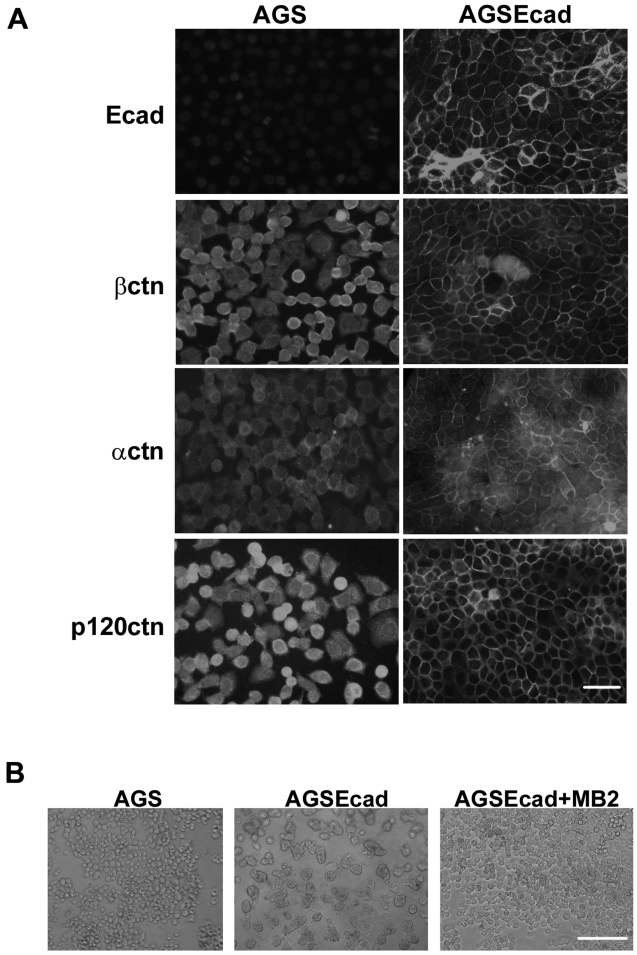


Figure 2. AGS cells stably expressing E-cadherin assemble a functional E-cadherin–catenin complex. *A,* E-cadherin (cad) or β-, α-, or p120-catenin (ctn) of AGS and AGSEcad cells. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole. Scale bar represents 50 μm. *B,* Aggregation assay of AGS, AGSEcad, and AGSEcad cells cultured with an anti–E-cadherin antibody (AGSEcad+MB2). Scale bar represents 100 μm. Figures are representative of 3 independent experiments.

was characterized by Western blot and immunocytochemistry for E-cadherin expression (figures 1*C* and 2*A*). Only clones with a homogeneous expression of E-cadherin at the cell membrane were selected. To exclude clonal dependency, all experiments reported were performed with 2 distinct clones (clones 1a and 4b). Immunocytochemistry of the E-cadherin–transduced AGS cells (AGSEcad) showed E-cadherin and β-, α-, and p120-catenins mostly located at the cell membrane in a honeycomb-like pattern (figure 2*A*), which suggests that E-cadherin recruits β-, α-, and p120-catenin to this site to form an adhesion complex.

To elucidate the functionality of the E-cadherin–catenin complex, we performed an aggregation assay that evaluates the ability of cells to aggregate in an E-cadherin–dependent manner. Our results provide evidence that AGS cells that do not express E-cadherin are not able to aggregate but remain as single cells (figure 2*B*). In contrast, AGSEcad cells form small aggregates, suggesting the presence of a functional E-cadherin–catenin complex. The fact that these cells are no longer able to aggregate in the presence of an antibody inhibiting E-cadherin–mediated cell-cell adhesion confirms that the E-cadherin–catenin complex of AGSEcad cells is functional. These experiments demonstrate that, in AGS transduced with E-cadherin, β-, α-, and p120-catenins are recruited to the membrane, where they assemble a functional adhesion complex.

E-cadherin counteracts* H. pylori*–mediated cell-invasive phenotype and* H. pylori*–mediated c-Met and p120-catenin tyrosine phosphorylation. To investigate whether E-cadherin was sufficient to inhibit *H. pylori*-mediated invasive phenotype, AGS, AGSEcad, and NCI-N87 cells were infected with *H. pylori* strains 26695 and 60190 and were used in Matrigel invasion assays. After infection, AGSEcad cells display significantly lower levels of invasion than do AGS cells (figure 3*A*). Similar results were obtained in infected NCI-N87 cells that endogenously express E-cadherin, suggesting that E-cadherin expression is sufficient to suppress *H. pylori*–mediated invasive phenotype.

Because the invasive phenotype induced by *H. pylori* in AGS cells requires c-Met phosphorylation [25], we investigated the effect of E-cadherin on the phosphorylation status of this receptor on infection. In addition, because p120-catenin tyrosine phosphorylation is modulated by E-cadherin [29], we also investigated the phosphorylation status of p120-catenin. Therefore, lysates of *H. pylori*–infected AGS and AGSEcad cells were immunoprecipitated with an antibody recognizing tyrosine-phosphorylated residues and were immunoblotted with anti–c-Met and anti–p120-catenin antibodies (figure 3*B*). The expression of c-Met and of p120-catenin is not altered by *H. pylori* in any of the cell lines. In AGS cells, *H. pylori* increases the phosphorylation level of c-Met and p120-catenin. In contrast, in AGSEcad cells, *H. pylori* decreases the phosphorylation levels of both proteins. These results were further confirmed in the NCI-N87 cell line, where tyrosine phosphorylation levels of c-Met and p120-catenin decreased after infection (figure 3*B*). Overall, these results suggest that E-cadherin suppresses *H. pylori*–mediated phosphorylation of c-Met and of p120-catenin.

H. pylori *affects the localization of elements of the E-cadherin–catenin complex.* Using confocal microscopy, we also analyzed the localization of the elements of the E-cadherin–catenin complex after infection. One hour after infection with *H. pylori,* we observed an increase in the intensity of membrane staining of E-cadherin and of p120-catenin (figure 4*A*) and in the nuclear levels of β-catenin (data not shown). No differences in α-catenin were observed.

Because CagA localizes to the inner surface of the plasma membrane after injection [9] and the membrane staining of E-cadherin and p120-catenin increased after infection (figure

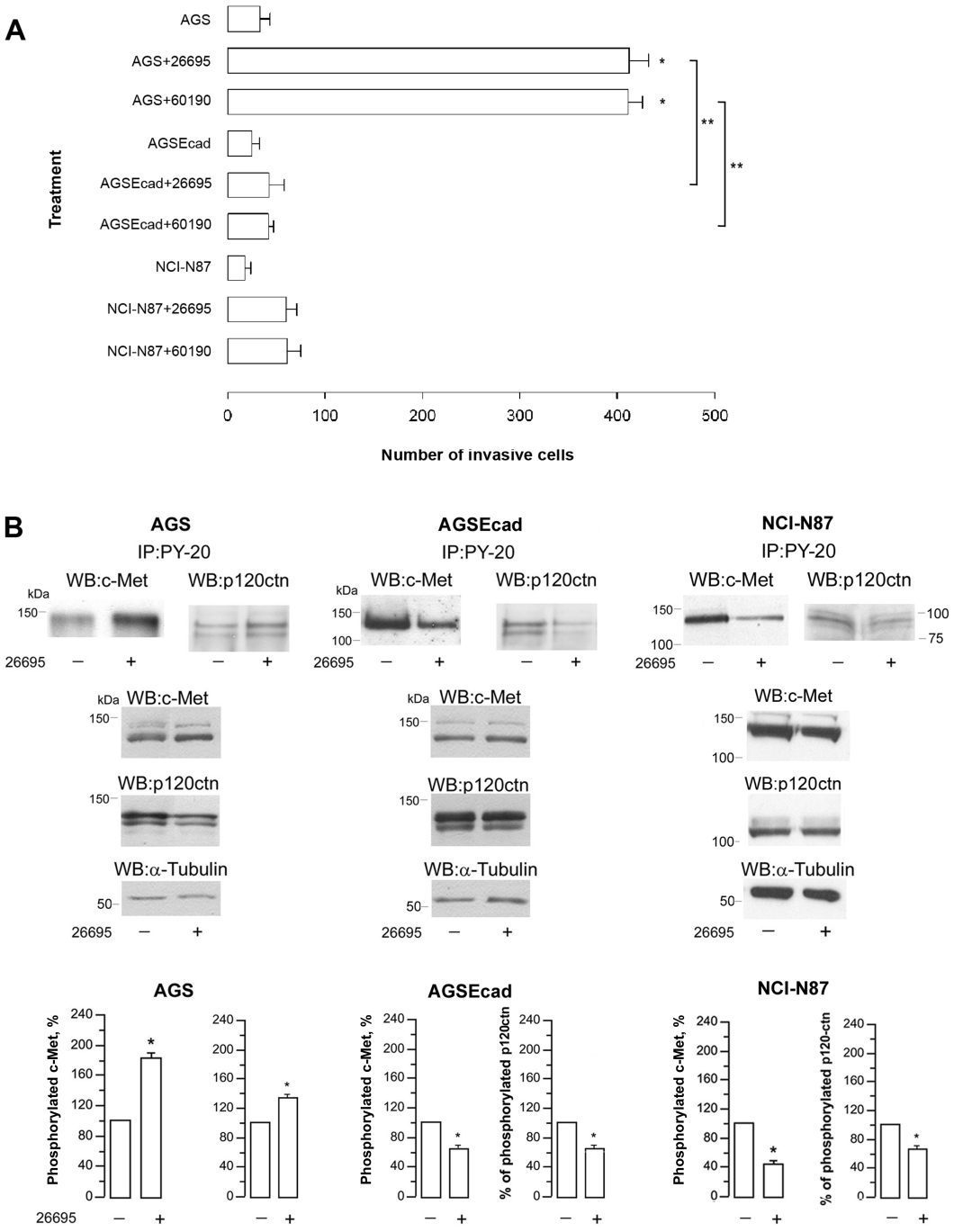


Figure 3. E-cadherin counteracts *Helicobacter pylori*–mediated cell-invasive phenotype, c-Met, and p120-catenin tyrosine phosphorylation. *A,* AGS, AGSEcad, and NCI-N87 cells infected or not infected for 24 h with *H. pylori* strain 26695 or 60190 on Matrigel-coated filters. Graphics represent the mean value of invasion ± standard deviation and are representative of 3 independent experiments. *B,* Western blot analysis of AGS, AGSEcad, and NCI-N87 cells infected with *H. pylori* for 1 h. Total cell lysates were immunoprecipitated with an antibody against tyrosine-phosphorylated residues (IP:PY-20) and immunostained with anti–c-Met (WB:c-Met) or anti-p120-catenin (WB:p120ctn) antibodies. In parallel, total cell lysates were immunostained with the same antibodies to control differences of expression. α-tubulin immunostaining was used as loading control. Graphics represent the variation in c-Met and p120-catenin tyrosine phosphorylation in comparison with the endogenous phosphorylation levels of uninfected cells. Data correspond to the mean values ± standard deviation and are representative of 3 independent experiments. *Significantly different from uninfected cells; **Significantly different from infected AGS cells.

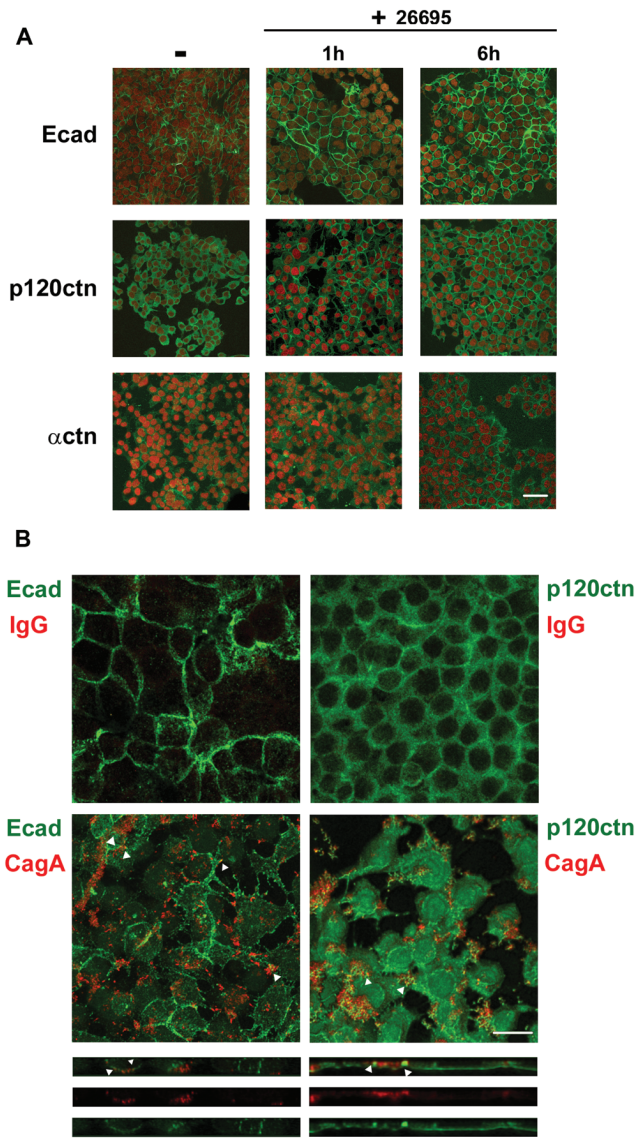


Figure 4. *Helicobacter pylori* affects the localization of elements of the E-cadherin–catenin complex. *A,* AGSEcad monolayers were infected with *H. pylori* for 1 or 6 h. After fixation, monolayers were stained with antibodies specific for E-cadherin (Ecad) or p120- or α-catenins (ctn; *green*). Nuclei were counterstained with 4′-6-diamidino-2-phenylindole *(red).* Scale bar represents 40 μm. Images are representative of 3 independent experiments. B, AGSEcad monolayers infected with *H. pylori* for 1 h were double immunostained for Ecad *(green)* and CagA *(red)* or p120ctn *(green)* and CagA *(red).* Immunostainings were also performed using a CagA isotype antibody as control. Xz or yz sections for both channels or for each channel apart are shown in more detail below each panel. Arrowheads indicate areas of colocalization. Images were obtained with a confocal microscope and are representative of 2 independent experiments. Scale bar represents 10 μm.

4*A*), we investigated putative colocalization between CagA and these E-cadherin–catenin complex elements. Analysis of the xz and yz confocal sections suggest that, after 1 h of infection of AGSEcad cells, CagA partially colocalizes with E-cadherin and with p120-catenin. An immunofluorescent signal corresponding to CagA was also visualized in areas negative for p120-catenin and E-cadherin (figure 4*B*). Specificity of the immunostaining was confirmed with nonimmunizing immunoglobulin G (IgG) of the same isotype.

H. pylori *CagA interacts with E-cadherin and with p120-catenin.* Because *H. pylori* CagA interacts and colocalizes with elements of the E-cadherin–catenin complex, we investigated the role of *H. pylori* virulence protein CagA in this interaction. Therefore, lysates of *H. pylori*–infected AGSEcad cells were immunoprecipitated with anti–E-cadherin or anti-p120 catenin antibodies and were immunoblotted with an anti-CagA antibody. We observed that CagA co-immunoprecipitates with E-cadherin, and of interest, we also observed that CagA co-immunoprecipitates with p120-catenin (figure 5*A*). Immunoprecipitation with nonimmunizing IgGs of the same isotype confirmed the specificity of such interactions. Likewise, in NCI-N87 cells, CagA co-imunoprecipitates with E-cadherin and also with p120-catenin (figure 5*A*). Furthermore, in the presence of bacteria in both AGSEcad and NCI-N87 cells, the binding between E-cadherin and p120-catenin is enhanced (figure 5*B* and 5*C*). No differences were observed in the interaction between β-catenin and α-catenin. Thus far, our results indicate that, after infection, *H. pylori* CagA binds to elements of the E-cadherin–catenin complex.

The interactions established between CagA and E-cadherin and between CagA and p120-catenin are mediated by c-Met. Because CagA targets the c-Met receptor, inducing its phosphorylation and activating downstream effector molecules [12], we examined whether the interactions between CagA and E-cadherin and between CagA and p120-catenin occurred downstream c-Met. Co-immunoprecipitation studies of *H. pylori*–infected AGSEcad cells confirmed that CagA interacts with c-Met (figure 5*A*), as described elsewhere [12]. Of interest, interactions established between c-Met and E-cadherin and between c-Met and p120-catenin are enhanced after infection with *H. pylori* 26695 and 60190 (figure 6*A* and 6*B*). In agreement with these observations, in *H. pylori*–infected NCI-N87 cells, increased binding of c-Met and E-cadherin and of c-Met and p120-catenin was also observed (figure 6*A*). When we infected cells with a *cagA* mutant strain (60190CagA−), there was a decrease in interactions between c-Met and E-cadherin but not in interactions between c-Met and p120-catenin (figure 6*B*); this suggests that CagA plays a role in the formation of the interaction between c-Met and E-cadherin.

Studies with siRNA targeting c-Met did not affect the expression of p120-catenin and, as expected, abolished the interactions between c-Met and p120-catenin (figure 6*C* and 6*D*) and those between c-Met and E-cadherin (data not shown). Of note, silencing of c-Met abolished the interactions between CagA and p120-catenin and also between CagA and E-cadherin

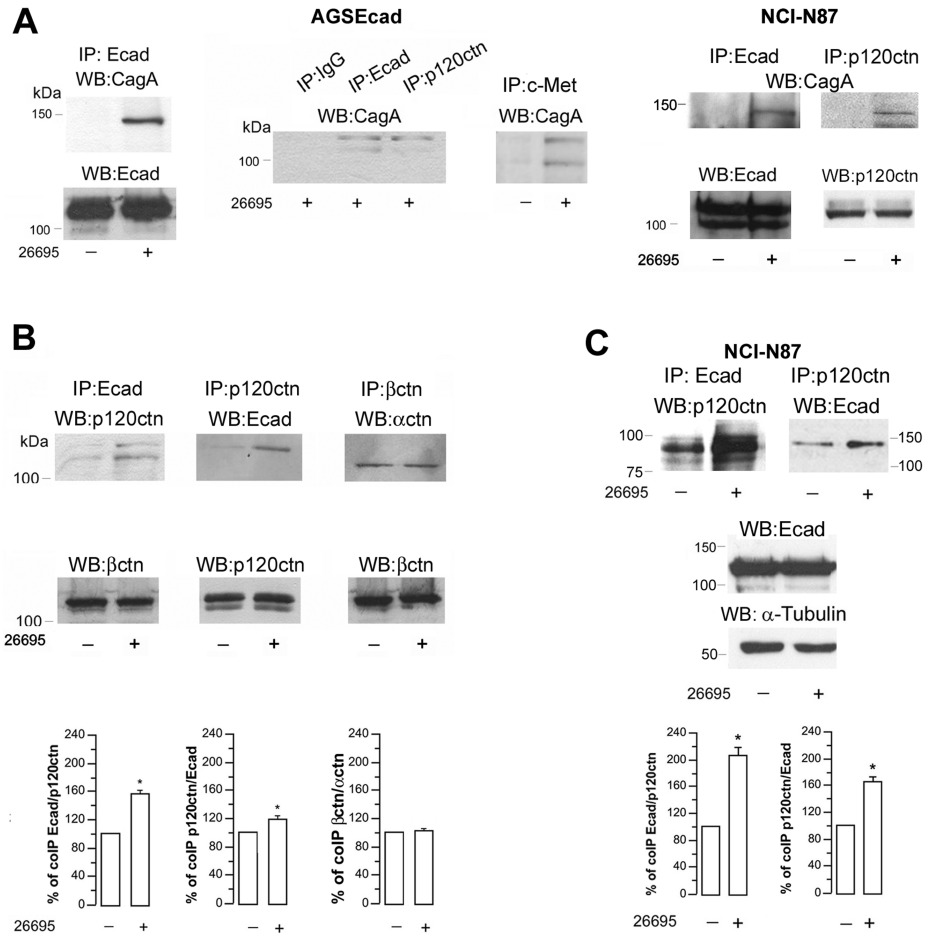


Figure 5. *Helicobacter pylori* CagA interacts with E-cadherin (Ecad), p120-catenin (ctn), and c-Met. *A,* AGSEcad and NCI-N87 cells were infected with *H. pylori* for 1 h. Cell lysates were immunoprecipitated with anti-Ecad, anti-p120ctn or anti-c-Met antibodies and were used for Western blot (WB) to be immunostained with an anti-CagA antibody. Immunoprecipitation (IP) was also performed with the same isotype control immunoglobulin G. Immunoblots were restained for Ecad or p120ctn as loading controls. B, AGSEcad cells were infected with *H. pylori* for 1 h. Cell lysates were immunoprecipitated with anti-Ecad, anti-p120ctn, or anti-β-ctn antibodies and were used for WB to be immunostained with anti-p120ctn, anti-Ecad, and anti-αctn antibodies. Immunoblots were restained for p120- or β-ctns as loading controls. *C,* NCI-N87 cells were infected with *H. pylori* for 1 h. Cell lysates were immunoprecipitated with anti-Ecad or anti-p120ctn antibodies and were used for WB to be immunostained with the same antibodies. Immunoblot was restained for Ecad as loading control. Graphics represent the variation in protein co-IP in comparison with the endogenous levels of uninfected cells. Data correspond to the mean values $\pm$ standard deviation and are representative of 3 independent experiments. *Significantly different from uninfected cells.

(figure 6*D*). These results suggest that interactions established between CagA and E-cadherin and between CagA and p120-catenin are c-Met dependent.

Altogether, our results evidence that *H. pylori* alters the localization of elements of the E-cadherin–catenin complex, leading to formation of a multiproteic complex composed by CagA, c-Met, E-cadherin, and p120-catenin. The formation of this complex impairs c-Met and p120-catenin tyrosine phosphorylation and suppresses the invasive phenotype induced by *H. pylori.*

DISCUSSION

In the present study, we demonstrated that E-cadherin is sufficient to suppress *H. pylori*–mediated cell invasion. We previously showed that *H. pylori* induces an invasive phenotype of AGS cells into extracellular matrix components [25]. In cell lines containing an intact E-cadherin–catenin complex, *H. pylori* induced lower levels or no invasion. By using 2 clones from an E-cadherin stably transduced cell line (AGSEcad), in which a functional E-cadherin–catenin complex was established, we have shown that E-cadherin is sufficient to suppress the invasive phenotype induced by *H. pylori.*

Induction of AGS-invasive phenotype by *H. pylori* involves c-Met tyrosine phosphorylation, as we described elsewhere [25]. In contrast, in E-cadherin–catenin functional cell lines, *H. pylori* decreased the phosphorylation levels of c-Met. Also, depending on the E-cadherin status, infection with *H. pylori* has different affects on the tyrosine phosphorylation of p120-catenin. In cells

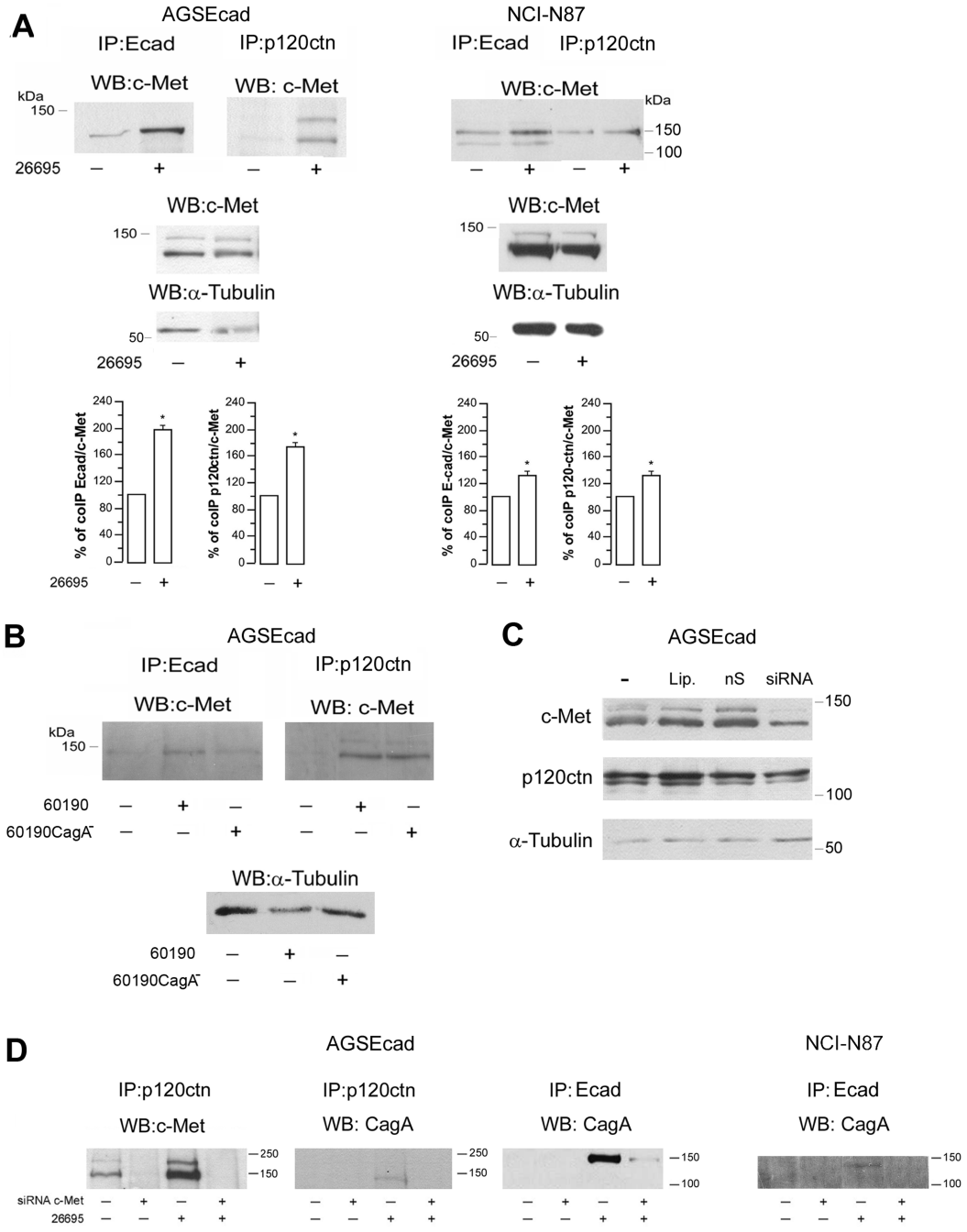


Figure 6. The interaction established between CagA, E-cadherin (Ecad), and p120-catenin (ctn) is mediated by c-Met. *A,* AGSEcad and NCI-N87 cells were infected with *Helicobacter pylori* for 1 h, and cell lysates were immunoprecipitated with anti-Ecad or anti-p120ctn antibodies and used for Western blot (WB) to be immunostained with an anti–c-Met antibody. α-Tubulin was used as a loading control. Graphics represent the variation in protein co-immunoprecipitation (IP) in comparison with the endogenous levels of uninfected cells. Data correspond to the mean value ± standard deviation and are representative of 3 independent experiments. *B,* AGSEcad cells were infected with *H. pylori* strain 60190 and with its *cagA* mutant (60190CagA-) for 1 h. Cell lysates were immunoprecipitated with anti-Ecad or anti-p120ctn antibodies and used for WB to be immunostained with an anti–c-Met antibody. Anti–c-Met and anti–α-tubulin antibodies were used as loading controls. *C,* AGSEcad cells were transiently transfected with small interfering RNA (siRNA) directed to c-Met. The effect of transfection on c-Met, p120-ctn, and α-tubulin expression was evaluated by WB with specific antibodies. *D,* AGSEcad and NCI-N87 cells transfected or not transfected with c-Met siRNA were infected with *H. pylori* for 1 h. Cell lysates were immunoprecipitated with anti-p120ctn and anti-Ecad antibodies and used on WB to be immunostained with anti–c-Met and anti-CagA antibodies. Data are representative of 2 independent experiments. Lip, lipofectamine; nS, nonsilencing siRNA.

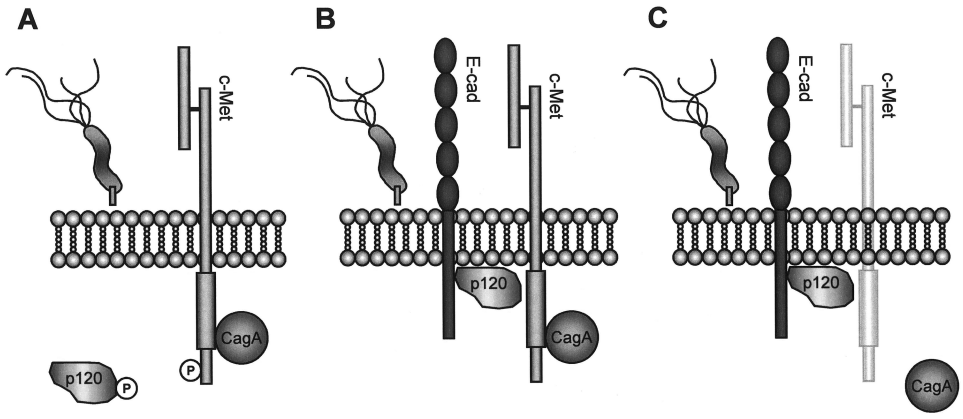


Figure 7. Hypothetical model of *Helicobacter pylori* CagA, c-Met, E-cadherin (E-cad), and p120-catenin interactions. *A,* In E-cad–negative cells, *H. pylori* CagA leads to an increase in the phosporylation levels of both c-Met and p120-catenin and to an invasive phenotype of cells. *B,* In cells with an intact E-cad–catenin complex *H. pylori* infection leads to enhanced interactions between c-Met and E-cad, c-Met and p120-catenin, and E-cad and p120-catenin. In these cells, CagA binds to c-Met, and this complex then interacts with the E-cad–catenin complex, suppressing *H. pylori*–mediated c-Met and p120-catenin phosphorylation and cell-invasive phenotype. *C,* c-Met is a crucial molecule in the establishment of the multiproteic complex.

lacking E-cadherin expression, *H. pylori* induced p120-catenin phosphorylation, whereas in cells expressing E-cadherin, *H. pylori* led to reduced p120-catenin phosphorylation. Taken together, our data point to a role for E-cadherin in the suppression of *H. pylori*–induced c-Met and p120-catenin phospory-lation and, consequently, in the suppression of the cell-invasive phenotype.

c-Met is a receptor tyrosine kinase with a well-documented participation in cell invasion [30–32]. Phosphorylation of tyrosine residues at the c-Met receptor intracellular domains results in the phosphorylation and binding of adaptor proteins and activation of signal transducers, eventually leading to cell invasion [32]. p120-Catenin interacts with the cytoplasmic juxtamembrane domain of E-cadherin [33], and this interaction and the phosphorylation status of p120-catenin play an important role in the stabilization of E-cadherin [14, 34]. Phosphorylation of p120-catenin promotes its dissociation from E-cadherin and its translocation to the cytoplasm or nucleus, where it participates in many signaling events [35]. Endogenous p120-catenin is described to promote migration and invasiveness of E-cadherin–deficient cells [34]. Our findings are in agreement with other experimental models in which re-establishment of E-cadherin function in E-cadherin–deficient cell lines reversed the invasive phenotype, pointing to a role for E-cadherin as a suppressor of cell invasion [26–28]. The present study constitutes, to our knowledge, the first experimental evidence implicating E-cadherin as a suppressor of bacterial-mediated cell-invasive phenotype.

In our experimental model, in both AGSEcad and NCI-N87 cells, E-cadherin expression levels were not altered by *H. pylori,* and this is concordant with previously published data in in vitro models [36, 37]. Although there is one description of an association between *H. pylori* infection and downregulation of E-cadherin expression [38], our findings are also in accordance with those of the vast majority of the studies performed using gastric biopsy specimens that show no association between the 2 events [36, 39–41]. In line with the observations of others, we found that *H. pylori* infection also did not affect the expression levels of p120-, β-, or α-catenins [36, 42]. Although no differences were observed in the expression of these molecules, *H. pylori* infection led to increased intensity in membrane staining of E-cadherin and of p120-catenin, as well as increased nuclear translocation of β-catenin. These experimental results concur with results of earlier research indicating increased nuclear levels of β-catenin after infection [24]. In keeping with increased intensity in E-cadherin and p120-catenin membrane staining is the experimental evidence from our immunoprecipitation studies, which revealed enhanced binding of E-cadherin and p120-catenin after infection, suggesting that *H. pylori* alters the organization of the E-cadherin–catenin complex.

One of the known effectors of *H. pylori* T4SS is CagA. CagA is injected into the host cell cytoplasm and localizes to the inner surface of the plasma membrane [9]. Our confocal immunostainings pointed to colocalization between CagA and E-cadherin and, most interestingly, to colocalization of CagA and p120-catenin at the cell membrane. The immunoprecipitation assays using in vitro infection revealed that CagA indeed physically interacts with E-cadherin, c-Met, and p120-catenin. The finding that CagA interacts with E-cadherin is in agreement with the work of Murata-Kamiya et al [11], who reported that, in CagA-transfected cells, CagA interacts with E-cadherin, destabilizing the binding of E-cadherin and β-catenin. Our findings also confirm previous observations of interaction between

CagA and c-Met [12] and add novelty to the field by showing interaction between CagA and p120-catenin.

Another interesting observation in this study was that, after *H. pylori* infection, interactions between c-Met and E-cadherin and between c-Met and p120-catenin were enhanced. Taken together with the CagA interactions results, these observations suggest that CagA, E-cadherin, p120-catenin, and c-Met interact with each other, possibly forming a multiproteic complex. Furthermore, experiments with siRNA targeting c-Met revealed that, in the absence of c-Met, CagA was no longer able to interact with E-cadherin nor with p120-catenin, suggesting that interactions of CagA with the 2 elements of the E-cadherin–catenin complex occur via c-Met.

Our hypothesis is that, after infection, injected CagA interacts with c-Met, and this complex then interacts with the E-cadherin–catenin complex, leading to the formation of a multiproteic complex (figure 7). This model is supported by our coimmunoprecipitation and siRNA results and might explain the reorganization of the E-cadherin–catenin complex observed by immunocytochemistry. Additional studies should be performed to elucidate how these 4 molecules interact within the multiproteic complex. There is now increasing evidence that E-cadherin participates in and regulates several signaling pathways via its extracellular domain [43]. The extracellular domain of E-cadherin establishes not only homophilic but also heterophilic interactions with tyrosine kinase receptors such as c-Met [16–18]. It may be possible that the interaction between c-Met and E-cadherin is established via their extracelullar domain and that *H. pylori* CagA interacts with both E-cadherin and p120-catenin via c-Met.

In conclusion, we suggest that *H. pylori* infection leads to formation of a multiproteic complex composed of CagA, c-Met, E-cadherin, and p120-catenin. The formation of this complex impairs c-Met and p120-catenin tyrosine phosphorylation and suppresses the invasive phenotype induced by*H. pylori.* However, in the absence of E-cadherin, *H. pylori* infection associates with an increased ability of the cells to invade. We do not think that the invasive phenotype observed in our in vitro cell model overlaps with tumor cell invasion, as observed during tumor progression in vivo. The latter is the result of a multistage process involving other events in addition to E-cadherin impairment; in fact it is arguable whether *H. pylori* would play a role in tumor invasion, because often, *H. pylori* is not present in full-blown gastric carcinoma. Instead, *H. pylori* infection might play an important role in selecting for survival gastric epithelial cells harboring E-cadherin inactivation (genetic or epigenetic) that would otherwise undergo apoptosis before actual tumor progression occurs. In other words, E-cadherin inactivation is an initiator event, whereas *H. pylori* infection works as a tumor promoter. As such, *H. pylori* infection would increase the likelihood of E-cadherin–deficient cell lineage survival and further accumulation of oncogenic events in a process that, in the early phase, becomes *H. pylori* independent.

Acknowledgments

We thank Prof. Marc Bracke, for the MB2 antibody, and Rachel Thomas, Richard Argent, and John C. Atherton, for the *Helicobacter pylori cagA* mutant strain.

References

1. Covacci A, Telford JL, Del Giudice G, Parsonnet J, Rappuoli R. *Helicobacter pylori* virulence and genetic geography. Science **1999**; 284: 1328–33.
2. Figueiredo C, Machado JC, Pharoah P, et al. *Helicobacter pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. J Natl Cancer Inst **2002**; 94:1680–7.
3. Peek RM, Blaser MJ. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer **2002**; 2:28–37.
4. Atherton JC, Cao P, Peek RM Jr, Tummuru MK, Blaser MJ, Cover TL. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori:* association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem **1995**; 270:17771–7.
5. Censini S, Lange C, Xiang Z, et al. cag, A pathogenicity island of *Helicobacter pylori,* encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci USA **1996**; 93:14648–53.
6. Cover TL, Blanke SR. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. Nat Rev Microbiol **2005**; 3:320–32.
7. Blaser MJ, Perez-Perez GI, Kleanthous H, et al. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res **1995**;55: 2111–5.
8. Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. Science **2000**; 287:1497–500.
9. Higashi H, Yokoyama K, Fujii Y, et al. EPIYA motif is a membrane-targeting signal of *Helicobacter pylori* virulence factor CagA in mammalian cells. J Biol Chem **2005**; 280:23130–7.
10. Amieva MR, Vogelmann R, Covacci A, Tompkins LS, Nelson WJ, Falkow S. Disruption of the epithelial apical-junctional complex by *Helicobacter pylori* CagA. Science **2003**; 300:1430–4.
11. Murata-Kamiya N, Kurashima Y, Teishikata Y, et al. *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the β-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene **2007**; 26:4617–26.
12. Churin Y, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. *Helicobacter pylori* CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol **2003**; 161:249–55.
13. Mengaud J, Ohayon H, Gounon P, Mège R-M, Cossart P. E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. Cell **1996**; 84:923–32.
14. Reynolds AB, Roczniak-Ferguson A. Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene **2004**; 23:7947–56.
15. Hoschuetzky H, Aberle H, Kemler R. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J Cell Biol **1994**; 127:1375–80.
16. Mateus AR, Seruca R, Machado JC, et al. EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells. Hum Mol Genet **2007**; 16:1639–47.
17. Hiscox S, Jiang WG. Association of the HGF/SF receptor, c-Met, with the cell-surface adhesion molecule, E-cadherin, and catenins in human tumor cells. Biochem Biophys Res Commun **1999**; 261:406–11.
18. Reshetnikova G, Troyanovsky S, Rimm DL. Definition of a direct extra-

cellular interaction between Met and E-cadherin. Cell Biol Int **2007**; 31: 366–73.
19. Anastasiadis PZ, Moon SY, Thoreson MA, et al. Inhibition of RhoA by p120 catenin. Nat Cell Biol **2000**; 2:637–44.
20. Guilford P, Hopkins J, Harraway J, et al. E-cadherin germline mutations in familial gastric cancer. Nature **1998**; 392:402–5.
21. Machado JC, Oliveira C, Carvalho R, et al. E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncogene **2001**; 20:1525–8.
22. Chan AO, Lam SK, Wong BC, et al. Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer. Gut **2003**; 52:502–6.
23. Leung WK, Man EP, Yu J, et al. Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res **2006**; 12:3216–21.
24. Franco AT, Israel DA, Washington MK, et al. Activation of beta-catenin by carcinogenic *Helicobacter pylori.* Proc Natl Acad Sci USA **2005**; 102: 10646–51.
25. Oliveira MJ, Costa AC, Costa AM, et al. *Helicobacter pylori* induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. J Biol Chem **2006**; 281:34888–96.
26. Suriano G, Oliveira C, Ferreira P, et al. Identification of CDH1 germline missense mutations associated with functional inactivation of the E-cadherin protein in young gastric cancer probands. Hum Mol Genet **2003**; 12:575–82.
27. Frixen UH, Behrens J, Sachs M, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol **1991**; 113:173–85.
28. Vleminckx K, Vakaet L Jr, Mareel M, Fiers W, van Roy F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion-suppressor role. Cell **1991**; 66:107–19.
29. Ozawa M, Ohkubo T. Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. J Cell Sci **2001**; 114:503–12.
30. Weidner KM, Behrens J, Vandekerckhove J, Birchmeier W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J Cell Biol **1990**; 111:2097–108.
31. Giordano S, Zhen Z, Medico E, Gaudino G, Galimi F, Comoglio PM. Transfer of motogenic and invasive response to scatter factor/hepatocyte growth factor by transfection of human MET protooncogene. Proc Natl Acad Sci USA **1993**; 90:649–53.
32. Trusolino L, Bertotti A, Comoglio PM. A signaling adapter function for alpha6beta4 integrin in the control of HGF-dependent invasive growth. Cell **2001**; 107:643–54.
33. Yap AS, Niessen CM, Gumbiner BM. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol **1998**; 141:779–89.
34. Yanagisawa M, Anastasiadis PZ. p120 Catenin is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness. J Cell Biol **2006**; 174:1087–96.
35. van Hengel J, Vanhoenacker P, Staes K, van Roy F. Nuclear localization of the p120ctn armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. Proc Natl Acad Sci USA **1999**; 96:7980–5.
36. Bebb JR, Leach L, Zaitoun A, et al. Effects of *Helicobacter pylori* on the cadherin-catenin complex. J Clin Pathol **2006**; 59:1261–6.
37. Conlin VS, Curtis SB, Zhao Y, et al. *Helicobacter pylori* infection targets adherens junction regulatory proteins and results in increased rates of migration in human gastric epithelial cells. Infect Immun **2004**; 72: 5181–92.
38. Terrés AM, Pajares JM, O'Toole D, Ahern S, Kelleher D. *H. pylori* infection is associated with downregulation of E-cadherin, a molecule involved in epithelial cell adhesion and proliferation control. J Clin Pathol **1998**; 51:410–2.
39. Zullo A, Romiti A, Borrini F, et al. Alteration of E-cadherin expression in gastric mucosa: role of intestinal metaplasia and *Helicobacter pylori* infection. Anticancer Res **2004**; 24:1603–7.
40. Shun CT, Wu MS, Lin MT, Chang MC, Lin JT, Chuang SM. Immunohistochemical evaluation of cadherin and catenin expression in early gastric carcinomas: correlation with clinicopathologic characteristics and *Helicobacter pylori* infection. Oncology **2001**; 60:339–45.
41. Chan AO, Wong BC, Lan HY, et al. Deregulation of E-cadherin-catenin complex in precancerous lesions of gastric adenocarcinoma. J Gastroenterol Hepatol **2003**; 18:534–9.
42. Krueger S, Hundertmark T, Kuester D, Kalinski T, Peitz U, Roessner A. *Helicobacter pylori* alters the distribution of ZO-1 and p120ctn in primary human gastric epithelial cells. Pathol Res Pract **2007**; 203: 433–44.
43. McLachlan RW, Yap AS. Not so simple: the complexity of phosphotyrosine signaling at cadherin adhesive contacts. J Mol Med **2007**; 85: 545–54.

Paper III

Adherens junctions as targets of microorganisms: a focus on Helicobacter pylori

Costa AM, Leite M, Figueiredo C.

Submitted for publication

Adherens junctions as targets of microorganisms: a focus on *Helicobacter pylori*

Angela Margarida Costa[1,2] , Marina Leite[1], Ceu Figueiredo[1,2,#]

[1]IPATIMUP - Institute of Molecular Pathology and Immunology of the University of Porto and [2]Department of Pathology, Faculty of Medicine of the University of Porto, Porto Portugal

Running title: *H. pylori* and the adherens junctions

#**Correspondence to**: **Ceu Figueiredo,** PhD., IPATIMUP, Rua Dr. Roberto Frias S/N 4200-465 Porto, Portugal. Tel. 351-22-5570700; Fax: 351-22-5570799; E-mail: cfigueiredo@ipatimup.pt

Abstract

Mucosal epithelia are targeted by several microorganisms as a way of adhesion, internalization, and/or exploitation of the host properties to induce disease.

Helicobacter pylori are worldwide prevalent bacteria that colonize the human stomach. Persistent infection of the gastric mucosa with *H. pylori* and concurrent chronic gastritis induced by the bacterium are risk factors for gastric carcinoma. The interactions at the *H. pylori*-epithelial interface are important to understand the pathogenesis of these bacteria and the host responses that contribute to carcinogenesis. Here, we provide an overview of the interactions between *H. pylori* and proteins of the adherens junctions and discuss the potential role of those interactions in gastric carcinoma development.

Key words: *Helicobacter pylori*, adherens junctions, infection, gastric carcinoma

Epithelial cell-cell junctions

Mucosal epithelia establish an interface between the external environment and the internal organs. They are physical and functional barriers that prevent external elements, like microorganisms, to reach the interstitial space and the blood stream. This is mainly accomplished by the existence of cell-cell junctions – tight junctions, adherens junctions, desmosomes, and gap junctions – which are essential components of epithelial integrity (57, 93).

The tight junctions are the most apical set of cell-cell junctions that separate the apical and the basolateral domains of the plasma membrane, and selectively regulate the paracellular flux of ions and small molecules (5). They are composed of transmembrane proteins occludin, claudins, and junctional adhesion molecules (JAMs), and cytosolic proteins, zona ocludens (ZO)-1, -2, and 3, that bridge transmembrane proteins with the cytoskeleton (92).

Adherens junctions are localized immediately below tight junctions and their main function is to maintain cell-cell adhesion. The major component of the adherens junctions is the transmembrane protein E-cadherin. The extracellular part of E-cadherin establishes homophilic interactions with E-cadherin molecules of neighboring cells, promoting cohesion of the epithelium. E-cadherin may also establish heterophilic interactions, namely with the receptor tyrosine kinases EGFR and c-Met (32, 33, 52, 76), modulating their signaling properties. The cytoplasmic domain of E-cadherin is associated with β-, p120-, and α-catenins, and the latter establishes a connection between E-cadherin and the actin cytoskeleton. These protein-protein interactions, as well as the phosphorylation status of the catenins, are important in junction stabilization (77, 106).

Desmosomes provide mechanical stability and intercellular communication to neighboring cells. They are composed by transmembrane desmoglein and desmocollin cadherins, which bind cytoplasmatic plakoglobin and plakophilin, which in turn bind to

desmoplakin. The latter anchors intermediate filaments, which establish a mechanical continuum across cells (40, 54, 66). Gap junctions are formed channels constituted by connexins, that allow intercellular passage of ions and small molecules. In addition, gap junctions also have a role in regulating cell morphology, establishing polarity, and rearrangement of the cytoskeleton (53).

Adherens junctions as targets of microorganisms

Disruption of the intercellular junctions is a strategy used by several microorganisms as a means of adhering to, entering cells, and/or exploiting host signaling to their advantage. Although the first line of defense against infectious agents in mucosal epithelia are tight junctions, microorganisms also explore cell-cell junctions at levels below the tight junctions. Indeed, E-cadherin is used as a receptor for adhesion and/or internalization by several microorganisms, allowing microbial persistence in the host, avoidance of mechanical clearance, and increased pathogenesis.

The best example of the use of E-cadherin for adhesion and internalization is the Gram-positive food-borne pathogen *Listeria monocytogenes*. *L. monocytogenes* has the ability to induce its internalization into non-phagocytic cells and to cross the intestinal, placental and blood-brain barriers (11). *L. monocytogenes* utilizes cell wall surface internalins A (InlA) and B (InlB) to promote adherence and internalization into host cells (55). InlA specifically interacts with E-cadherin and recruits α-, β- and p120-catenins, as well as actin, to the site of bacterial entry (47, 48).

The Als3 adhesin of the opportunistic fungus *Candida albicans* mimics host cell cadherins and induces fungal endocytosis by adhering to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells (73). This mechanism is important for hematogenously disseminated and oropharyngeal candidiasis, characterized by fungal invasion of host cells.

Streptococcus pneumoniae adheres to the epithelial cell surface in the first steps of nasopharyngeal carriage and colonization, and E-cadherin has been identified as a receptor for the pneumococcal surface adhesin A (PsaA) (3).

E-cadherin has also been reported as a target of bacterial proteases. E-cadherin cleavage leads to weaker cell-cell adhesion which may allow access of the microorganisms to the intercellular epithelial spaces and to the tissues underlying epithelia.

Enterotoxigenic *Bacteroides fragilis* produce a toxin named fragilysin or BFT, which specifically cleaves the extracellular domain of E-cadherin on intestinal and other polarized epithelial cells, resulting in junction disassembly (101). E-cadherin cleavage by BFT releases β-catenin to the cytoplasm, resulting in β-catenin nuclear localization and stimulation of β-catenin-TCF-dependent cell proliferation (102). This BFT-mediated extracellular domain cleavage of E-cadherin also induces proteolysis of intracellular E-cadherin, in a process dependent on γ-secretase (103).

Porphyromonas gingivalis, an etiological agent of human adult periodontitis, produces cysteine proteases termed gingipains, namely HRgpA, RgpB, and Kgp, which degrade E-cadherin. This process of disruption of the adherens junctions suggests that *P. gingivalis* can invade the underlying connective tissues via a paracellular pathway (41).

Enterococcus faecalis are commensals of the mammalian gastrointestinal tract, which can cause opportunistic infections when they penetrate the gut barrier. The GelE metalloprotease from *E. faecalis* cleaves the extracellular domain of E-cadherin, and is implicated in the development of chronic intestinal inflammation by impairment of the epithelial barrier integrity. Mice that are susceptible to intestinal inflammation show significantly reduced levels of the extracellular domain of E-cadherin, which result from GelE-mediated proteolytic cleavage (83).

The contact of *C. albicans* with oral and with intestinal epithelial cells induces E-cadherin cleavage, both in the extracellular and in the intracellular domains, and γ-

secretase is implicated in the latter cleavage event (27, 97). Interestingly, during the interaction of *C. albicans* with oral epithelial cells, E-cadherin is degraded only in localized areas of tissue invasion by the Sap5p secreted aspartyl-protease of the fungus (97).

Staphylococcus aureus contributes to the pathogenesis of pneumonia by secreting α-hemolysin (Hla). Hla induces injury in epithelial cells by interacting with its receptor, the zinc-dependent metalloprotease ADAM10. Hla upregulates ADAM10 metalloprotease activity in alveolar epithelial cells, resulting in cleavage of E-cadherin. E-cadherin cleavage is associated with disruption of epithelial barrier function, contributing to the pathogenesis of lethal acute lung injury (39).

Additional mechanisms of pathogen-induced adherens junctions disruption that do not require E-cadherin cleavage have also been described. *Clostridium botulinum* produces the botulinum neurotoxin (BoNT), the etiological agent of botulism, which forms large protein complexes through associations with non-toxic components, such as hemagglutinin (HA). HA directly binds E-cadherin and disrupts E-cadherin-mediated cell-cell adhesion and the epithelial barrier, in a mechanism that does not include proteolysis of E-cadherin (86).

Pseudomonas aeruginosa disrupts adherens junctions by altering the phosphorylation status of E-cadherin and β-catenin, which is paralleled by changes in the expression and distribution of these junctional proteins (96).

Disruption of the adherens junctions by *Helicobacter pylori*

Helicobacter pylori are spiral-shaped Gram-negative bacteria colonize the stomach of more than half of the world population (10, 58). *H. pylori* infection results in chronic gastritis in all infected individuals, a condition that persists during the host lifetime if the infection is not eradicated. Infection may also result in more severe clinical outcomes, such as peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and

gastric carcinoma (85), and the diversity in virulence of *H. pylori* is an important factor in the infection outcome (18, 20, 50, 56, 80).

One of the most important differences in virulence between *H. pylori* strains is the presence of the *cag* pathogenicity island, encoding a type IV secretion system (T4SS), which allows translocation of the bacterial effector CagA into the host cells (10). Inside the host cell, CagA may undergo tyrosine phosphorylation by host kinases (65, 81, 90), and both phosphorylated and non-phosphorylated CagA can activate multiple signaling pathways leading to cell proliferation, cytoskeletal rearrangements, and disruption of cell-cell junctions (2, 90). Individuals infected with CagA-positive strains have higher degrees of inflammation and epithelial damage in the stomach, and enhanced risk for peptic ulcer disease and gastric carcinoma, than those infected with CagA-negative strains (4, 17, 70), especially those infected with strains harboring high number of tyrosine phosphorylation sites in CagA (6, 7, 24).

Several lines of evidence show that *H. pylori* interferes with cell-cell junctional complexes, although the importance of this phenomenon for gastric disease development is not fully understood.

In the gastric mucosa, *H. pylori* can be found within the mucus layer, and in close contact with the epithelial cells, preferentially at the apical side of the intercellular contacts (31, 62, 91). The tropism of the bacteria for this localization may contribute to alterations to the structure and function of the tight junctions. Indeed, it has been shown that *H. pylori* infection of epithelial cells induces internalization of occludin and claudins, and increases myosin light chain phosphorylation, leading to tight junction relaxation and increased epithelial barrier permeability (23, 46, 100). Further, CagA interacts with ZO-1 and JAM-A, and recruits these proteins to the sites of bacteria attachment, altering the composition and function of the tight-junctional complex (2).

Although not so frequently described in the literature, it has also been observed that *H. pylori* reaches the intercellular spaces below the tight junctions, and even penetrates the epithelium towards the *lamina propria* in the basolateral side of the cells (62), showing that *H. pylori* is able to disrupt the adherens junctions. Interestingly, there are also reports demonstrating the presence of *H. pylori* inside the host cells, both in biopsies and in *in vitro* models (21, 22, 62, 63, 72, 84, 99). Whether this particular localization of *H. pylori* is related to use the adherens junctions as a means of internalization, as it has been shown for other microorganisms, remains to be elucidated.

The known mechanisms underlying *H. pylori*-mediated adherens junctions disruption are diverse and directed towards different components of the complex.

Helicobacter pylori and E-cadherin

Studies have shown that *H. pylori* targets E-cadherin by several mechanisms. Promoter methylation of the *CDH1* gene (encoding E-cadherin) is frequently observed in *H. pylori*-infected individuals (12, 49, 71). The importance of this epigenetic mechanism of gene silencing in the context of *H. pylori* infection is reinforced by studies showing that eradication of *H. pylori* reduces *CDH1* promoter methylation levels (13, 49, 71). Although the molecular mechanisms through which *H. pylori* mediates E-cadherin promoter methylation are not fully understood, Qian *et al*. have shown that *CDH1* promoter methylation could be induced by infection with *H. pylori* or treatment of gastric cancer cell lines with interleukin-1β (IL-1β), a cytokine up-regulated in the context of *H. pylori* infection (75). This effect was inhibited by treatment with an interleukin-1 receptor antagonist (IL-1ra) antibody, suggesting that IL-1β may play a role in E-cadherin methylation (75). More recently, the same group has extended the study and showed that *H. pylori*- and IL-1β-mediated *CDH1* promoter methylation, led to decreased E-cadherin expression and concomitant increase in DNA

methyltransferase activity (37). Nevertheless, other studies have failed to show a decrease in E-cadherin expression associated with *H. pylori* infection both in cell lines and in the gastric mucosa (8, 12, 15). Of note, no *CDH1* mutations have been described associated to *H. pylori* infection.

In addition to epigenetic silencing, *H. pylori* has been associated with other mechanisms of disturbance of E-cadherin functions, such as proteolytic cleavage of its extracellular domain (also known as ectodomain shedding), and protein delocalization from the cell membrane.

Weidig *et al.* and Schirmeister *et al.* reported E-cadherin ectodomain shedding upon *H. pylori* infection in two different cell line models, the breast cancer MCF-7 and the gastric cancer NCI-N87 cells, respectively (79, 98). In both reports, ectodomain shedding of E-cadherin was neither dependent on *H. pylori* CagA nor on the presence of a functional T4SS. This finding is in keeping with a recent report showing that *H. pylori*-positive patients had significantly higher serum levels of soluble E-cadherin than uninfected controls, independently of the CagA status of the infecting strain (64). There is evidence that the host disintegrin metalloproteinase ADAM10 contributes to *H. pylori*-induced shedding of E-cadherin in NCI-N87 cells, but since specific inhibition of ADAM10 led to a partial inhibition of E-cadherin shedding, it is possible that other proteases are also involved in this process (79). In fact, Hoy *et al.* have recently identified the high-temperature requirement A (HtrA), a serine protease from *H. pylori*, as a new secreted virulence factor which cleaves the ectodomain of the E-cadherin. E-cadherin shedding by HtrA leads to epithelial barrier disruption and may allow *H. pylori* to access the intercellular spaces (35). HtrA-mediated E-cadherin cleavage may be a pathogenic mechanism of multiple Gram-negative bacteria, as is has recently been shown for pathogens such as enteropathogenic *Escherichia coli*, *Shigella flexneri*, and *Campylobacter jejuni* (34).

Studies have shown that *H. pylori* also induces E-cadherin translocation from the cell membrane to the cytoplasm (15, 79). Conlin *et al*. have demonstrated that *H. pylori*-induced redistribution of E-cadherin to intracellular vesicles was accompanied by the translocation from the cytoplasm to intracellular tubular structures of IQGAP-1, a protein that regulates the formation of adherens junctions. The results of E-cadherin internalization mediated by *H. pylori* in the NCI-N87 gastric cell line were confirmed in primary gastric cells. After 48 hours of bacterial infection, the presence of a reduced level of E-cadherin at the cell membrane was demonstrated, mediating a reduced level of cell-cell adhesion. These alterations to epithelial cell adhesion molecules induced by *H. pylori* were paralleled by increased levels of Rho-GTP and cell migration (15). Using the human breast cancer MCF-7 cell line, Weidig *et al.* also reported internalization of E-cadherin upon *H. pylori* infection, simultaneously with E-cadherin cleavage (98). A rapid dissociation of the E-cadherin/β-catenin/p120 complex from the actin cytoskeleton was observed upon infection, by disruption of the interaction between E-cadherin and α-catenin (98).

Although E-cadherin alterations mediated by *H. pylori* are independent of CagA, physical interaction between these proteins has been described by two independent groups using different cell lines and methodologies (59, 69). Murata-Kamiya *et al.* used the gastric cancer cell line MKN28 transfected with CagA expression vectors, and showed that CagA was able to interact with E-cadherin. This led to β-catenin release from the adherens junctions and nuclear translocation, with transactivation of genes encoding intestinal specific proteins like MUC2, contributing to the development of intestinal metaplasia (59). This effect was independent of the CagA tyrosine phosphorylation status but dependent on a specific region of CagA known as the multimerization sequence (45).

Using *in vitro* infection with live bacteria, Oliveira *et al*. showed that CagA interacts with E-cadherin, both in human gastric AGS cells transfected with wild type E-cadherin, and

in the NCI-N87 cell line that naturally establishes adherens junctions (69). Furthermore, and in parallel with CagA/E-cadherin interaction, interactions between p120 and CagA, and between c-Met and CagA were also observed after *H. pylori* infection, raising the possibility that the CagA/E-cadherin interaction might not be direct (69). In fact, silencing of c-Met in these cells abolished the interactions between CagA and E-cadherin, and CagA and p120, suggesting that CagA interaction with elements of the adherens junctions is mediated by c-Met (69). The CagA, c-Met, E-cadherin, and p120 multiproteic complex led to impairment of *H. pylori*-induced c-Met-mediated signaling and suppression of cell invasion (69). These findings are in agreement with previous observations in E-cadherin defective gastric cells in which *H. pylori* activated c-Met signaling (14, 68), and increased the activity of MMP2 and MMP9, leading to extracellular matrix degradation and subsequent cell invasion (68).

Helicobacter pylori and β-catenin

The binding of catenins to the intracellular domain of E-cadherin has a crucial role in the adherens junctional complex stabilization, connection to the cell cytoskeleton, and cell signaling.

When not tethered at the membrane in the adherens junctions, β-catenin is phosphorylated by a multiproteic complex comprising the serine/threonine kinase glycogen synthase kinase 3β (GSK3β), and the scaffolding proteins adenomatous polyposis coli (APC), axin, and casein kinase 1α (Ck1α) and targeted to degradation via the ubiquitin/proteasome pathway (1, 29). This degradation pathway is counteracted when the Wnt pathway is active, resulting in β-catenin stabilization in the cytoplasm. Cytoplasmic stabilized β-catenin may be translocated into the nucleus where it forms a nuclear complex with the transcription factors of the T cell factor/lymphoid enhancer-binding factor (TCF/LEF) family, promoting the expression of a wide-range of genes important for carcinogenesis (9, 29, 51, 74).

Several studies have addressed the effect of *H. pylori* infection in β-catenin signaling and have shown that *H. pylori* leads to the delocalization of β-catenin from the cell membrane (8, 25, 26, 28, 38, 45, 59, 61, 82, 87, 88, 98). *H. pylori* phosphorylates and inactivates GSK3β (61, 82, 87, 89) and, by suppressing GSK3β activity *H. pylori* leads to inhibition of β-catenin phosphorylation and ubiquitin-dependent degradation (82). *H. pylori*-mediated GSK3β suppression occurs through activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K) ⁄ (Akt) signaling pathway *via* EGFR and c-Met receptor (60, 61, 82, 87, 89, 105). In one report however, β-catenin stabilization was described to occur in a phosphorylation-independent process (25).

The canonical Wnt-signaling pathway appears to be involved in the regulation of β-catenin in the context of *H. pylori* infection. In fact, it has been shown that after *H. pylori* infection there is increased synthesis of Wnt10A by the epithelial cells (43). Additionally, *H. pylori*-induced activation of β-catenin was shown to involve the phosphorylation of the Wnt pathway co-receptor low density lipoprotein receptor-related protein LRP6 and proteins of the dishevelled family, namely Dvl2 and Dvl3 (28).

Several studies report β-catenin translocation to the nucleus after *H. pylori* infection (25, 26, 28, 38, 59, 61, 82, 87, 88). There are however two studies in non-gastric cell lines that did not show nuclear translocation or activation of β-catenin signaling after infection (8, 67). β-catenin nuclear translocation induced by *H. pylori* leads to TCF/LEF-dependent transcription of genes involved in carcinogenesis, including cyclin D1 (28, 45, 59, 61, 82). This effect was shown to be CagA-dependent in the majority of studies (25, 38, 45, 59, 87, 88), with the exception of one study performed in a non-human, non-gastric cell line (82). Additional effectors of the T4SS, like peptidoglycan (60, 95), and other *H. pylori* virulence factors, such as the multifunctional toxin VacA (16, 61) and the outer membrane protein OipA (26, 104), may also be involved. The use of the Mongolian gerbil model has shown that *H. pylori* infection induces gastric carcinoma precursor lesions, with aberrant β-catenin expression, that led to transcriptional up-

regulation of genes implicated in carcinogenesis. In this model-system, and in agreement with most of the cell-line models, aberrant β-catenin expression was associated with infection with *H. pylori* CagA-positive strains (25, 26).

Helicobacter pylori and p120-catenin

p120-catenin (p120) is another important component in adherens junctional complex stability and signaling (30). p120 binds to the juxtamembrane domain of E-cadherin (107), and mutations in this domain abolish E-cadherin-mediated cell-cell adhesion (42). In addition to E-cadherin stabilization, p120 can also interact with the transcription factor Kaiso (19, 78, 94).

Two reports show that *H. pylori* alters the cellular localization and the phosphorylation status of p120, but not total p120 protein levels (44, 67). Krueger *et al.* showed that after six hours of infection of primary gastric epithelial cells, there was a recruitment of the non-phosphorylated p120 to perinuclear vesicles, whereas the fraction of phosphorylated p120 increased and could be detected in the nucleus, at the cell membrane, and at the leading edge of migrating cells (44). Those alterations were associated with elongation of cells and increased migration. Ogden *et al.* also showed nuclear translocation of p120 after *H. pylori* infection of MKN28 cells and of *ex vivo* mouse gastric glands. Nuclear translocation of p120 induced by *H. pylori* was associated with increased MMP-7 mRNA, and occurred by release of the MMP-7 transcriptional repressor Kaiso. These changes were associated with reduced levels of p120 tyrosine phosphorylation, while total p120 remained unchanged (67). *H. pylori*-mediated p120 nuclear translocation was dependent on factors of the T4SS, but independent of the T4SS effector CagA (67).

Relevance of H. pylori-adherens junctions interactions for gastric carcinoma development

When addressing the issue of *H. pylori* infection in cell-cell junction dysfunction two main aspects should be taken into consideration. First, a lot of studies have been performed in cell line models, often non-human and non-gastric, and frequently cancer-derived and with impaired cell-cell junctions. For example, the AGS and the MKN45 cell lines harbor mutations in the E-cadherin gene (108). Also, genetic alterations in components of the Wnt signaling pathway are common in cell lines such as MKN7, MKN28 and HT29 (36, 108). In addition, in host cell lines-bacteria co-culture models, infections cannot easily be maintained for long time periods, making these models more close to acute that to chronic infections.

Second, results obtained in cell lines do not match those observed in the gastric mucosa of *H. pylori*-infected patients. In particular, gastric biopsy specimens of patients with and without *H. pylori* infection do not show any relationship between the infection and changes in the expression or distribution of E-cadherin, β-catenin, or p120. This does not mean that it the phenomena observed *in vitro* do not occur *in vivo,* but may be explained if *H. pylori*-induced alterations in the adherens junctions are fast and transient and, therefore, not observable in fixed gastric tissues. Another possible explanation is that alterations in adherens junctions induced by *H. pylori* only occur in a subset of cells. These cells may then be selected as having growth advantages or increased resistance to apoptosis, therefore rendering them more susceptible to accumulation of DNA lesions which may lead to malignant transformation.

References

1. **Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler.** 1997. beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J **16:**3797-3804.
2. **Amieva, M. R., R. Vogelmann, A. Covacci, L. S. Tompkins, W. J. Nelson, and S. Falkow.** 2003. Disruption of the epithelial apical-junctional complex by Helicobacter pylori CagA. Science **300:**1430-1434.
3. **Anderton, J. M., G. Rajam, S. Romero-Steiner, S. Summer, A. P. Kowalczyk, G. M. Carlone, J. S. Sampson, and E. W. Ades.** 2007. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. Microb Pathog **42:**225-236.
4. **Atherton, J. C., K. T. Tham, R. M. Peek, Jr., T. L. Cover, and M. J. Blaser.** 1996. Density of Helicobacter pylori infection in vivo as assessed by quantitative culture and histology. J Infect Dis **174:**552-556.
5. **Balda, M. S., and K. Matter.** 2008. Tight junctions at a glance. J Cell Sci **121:**3677-3682.
6. **Basso, D., C. F. Zambon, D. P. Letley, A. Stranges, A. Marchet, J. L. Rhead, S. Schiavon, G. Guariso, M. Ceroti, D. Nitti, M. Rugge, M. Plebani, and J. C. Atherton.** 2008. Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. Gastroenterology **135:**91-99.
7. **Batista, S. A., G. A. Rocha, A. M. Rocha, I. E. Saraiva, M. M. Cabral, R. C. Oliveira, and D. M. Queiroz.** 2011. Higher number of Helicobacter pylori CagA EPIYA C phosphorylation sites increases the risk of gastric cancer, but not duodenal ulcer. BMC Microbiol **11:**61.
8. **Bebb, J. R., L. Leach, A. Zaitoun, N. Hand, D. P. Letley, R. Thomas, and J. C. Atherton.** 2006. Effects of Helicobacter pylori on the cadherin-catenin complex. J Clin Pathol **59:**1261-1266.
9. **Bierie, B., M. Nozawa, J. P. Renou, J. M. Shillingford, F. Morgan, T. Oka, M. M. Taketo, R. D. Cardiff, K. Miyoshi, K. U. Wagner, G. W. Robinson, and L. Hennighausen.** 2003. Activation of beta-catenin in prostate epithelium induces hyperplasias and squamous transdifferentiation. Oncogene **22:**3875-3887.
10. **Blaser, M. J., and J. C. Atherton.** 2004. Helicobacter pylori persistence: biology and disease. J Clin Invest **113:**321-333.
11. **Bonazzi, M., M. Lecuit, and P. Cossart.** 2009. Listeria monocytogenes internalin and E-cadherin: from bench to bedside. Cold Spring Harb Perspect Biol **1:**a003087.
12. **Chan, A. O., S. K. Lam, B. C. Wong, W. M. Wong, M. F. Yuen, Y. H. Yeung, W. M. Hui, A. Rashid, and Y. L. Kwong.** 2003. Promoter methylation of E-cadherin gene in gastric mucosa associated with Helicobacter pylori infection and in gastric cancer. Gut **52:**502-506.
13. **Chan, A. O., J. Z. Peng, S. K. Lam, K. C. Lai, M. F. Yuen, H. K. Cheung, Y. L. Kwong, A. Rashid, C. K. Chan, and B. C. Wong.** 2006. Eradication of Helicobacter pylori infection reverses E-cadherin promoter hypermethylation. Gut **55:**463-468.
14. **Churin, Y., L. Al-Ghoul, O. Kepp, T. F. Meyer, W. Birchmeier, and M. Naumann.** 2003. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. J Cell Biol **161:**249-255.
15. **Conlin, V. S., S. B. Curtis, Y. Zhao, E. D. Moore, V. C. Smith, R. M. Meloche, B. B. Finlay, and A. M. Buchan.** 2004. Helicobacter pylori infection targets adherens junction regulatory proteins and results in increased rates of migration in human gastric epithelial cells. Infect Immun **72:**5181-5192.
16. **Cover, T. L., and S. R. Blanke.** 2005. Helicobacter pylori VacA, a paradigm for toxin multifunctionality. Nature reviews. Microbiology **3:**320-332.

17. **Crabtree, J. E., T. M. Shallcross, R. V. Heatley, and J. I. Wyatt.** 1991. Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. Gut **32:**1473-1477.
18. **Crabtree, J. E., Z. Xiang, I. J. Lindley, D. S. Tompkins, R. Rappuoli, and A. Covacci.** 1995. Induction of interleukin-8 secretion from gastric epithelial cells by a cagA negative isogenic mutant of Helicobacter pylori. J Clin Pathol **48:**967-969.
19. **Daniel, J. M., and A. B. Reynolds.** 1999. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol **19:**3614-3623.
20. **El-Omar, E. M., M. Carrington, W. H. Chow, K. E. McColl, J. H. Bream, H. A. Young, J. Herrera, J. Lissowska, C. C. Yuan, N. Rothman, G. Lanyon, M. Martin, J. F. Fraumeni, Jr., and C. S. Rabkin.** 2000. Interleukin-1 polymorphisms associated with increased risk of gastric cancer. Nature **404:**398-402.
21. **el-Shoura, S. M.** 1995. Helicobacter pylori: I. Ultrastructural sequences of adherence, attachment, and penetration into the gastric mucosa. Ultrastructural pathology **19:**323-333.
22. **Engstrand, L., D. Graham, A. Scheynius, R. M. Genta, and F. El-Zaatari.** 1997. Is the sanctuary where Helicobacter pylori avoids antibacterial treatment intracellular? American journal of clinical pathology **108:**504-509.
23. **Fedwick, J. P., T. K. Lapointe, J. B. Meddings, P. M. Sherman, and A. G. Buret.** 2005. Helicobacter pylori activates myosin light-chain kinase to disrupt claudin-4 and claudin-5 and increase epithelial permeability. Infect Immun **73:**7844-7852.
24. **Ferreira, R. M., J. C. Machado, M. Leite, F. Carneiro, and C. Figueiredo.** 2012. The number of Helicobacter pylori CagA EPIYA C tyrosine phosphorylation motifs influences the pattern of gastritis and the development of gastric carcinoma. Histopathology **60:**992-998.
25. **Franco, A. T., D. A. Israel, M. K. Washington, U. Krishna, J. G. Fox, A. B. Rogers, A. S. Neish, L. Collier-Hyams, G. I. Perez-Perez, M. Hatakeyama, R. Whitehead, K. Gaus, D. P. O'Brien, J. Romero-Gallo, and R. M. Peek, Jr.** 2005. Activation of beta-catenin by carcinogenic Helicobacter pylori. Proc Natl Acad Sci U S A **102:**10646-10651.
26. **Franco, A. T., E. Johnston, U. Krishna, Y. Yamaoka, D. A. Israel, T. A. Nagy, L. E. Wroblewski, M. B. Piazuelo, P. Correa, and R. M. Peek, Jr.** 2008. Regulation of gastric carcinogenesis by Helicobacter pylori virulence factors. Cancer Res **68:**379-387.
27. **Frank, C. F., and M. K. Hostetter.** 2007. Cleavage of E-cadherin: a mechanism for disruption of the intestinal epithelial barrier by Candida albicans. Transl Res **149:**211-222.
28. **Gnad, T., M. Feoktistova, M. Leverkus, U. Lendeckel, and M. Naumann.** 2010. Helicobacter pylori-induced activation of beta-catenin involves low density lipoprotein receptor-related protein 6 and Dishevelled. Mol Cancer **9:**31.
29. **Gooding, J. M., K. L. Yap, and M. Ikura.** 2004. The cadherin-catenin complex as a focal point of cell adhesion and signalling: new insights from three-dimensional structures. Bioessays **26:**497-511.
30. **Hartsock, A., and W. J. Nelson.** 2008. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta **1778:**660-669.
31. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. Campylobacter pyloridis and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J Infect Dis **153:**658-663.
32. **Hiscox, S., and W. G. Jiang.** 1999. Association of the HGF/SF receptor, c-met, with the cell-surface adhesion molecule, E-cadherin, and catenins in human tumor cells. Biochem Biophys Res Commun **261:**406-411.

33. **Hoschuetzky, H., H. Aberle, and R. Kemler.** 1994. Beta-catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. J Cell Biol **127:**1375-1380.
34. **Hoy, B., T. Geppert, M. Boehm, F. Reisen, P. Plattner, G. Gadermaier, N. Sewald, F. Ferreira, P. Briza, G. Schneider, S. Backert, and S. Wessler.** 2012. Distinct roles of secreted HtrA proteases from gram-negative pathogens in cleaving the junctional protein and tumor suppressor E-cadherin. J Biol Chem **287:**10115-10120.
35. **Hoy, B., M. Lower, C. Weydig, G. Carra, N. Tegtmeyer, T. Geppert, P. Schroder, N. Sewald, S. Backert, G. Schneider, and S. Wessler.** 2010. Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion. EMBO Rep **11:**798-804.
36. **Hsi, L. C., J. Angerman-Stewart, and T. E. Eling.** 1999. Introduction of full-length APC modulates cyclooxygenase-2 expression in HT-29 human colorectal carcinoma cells at the translational level. Carcinogenesis **20:**2045-2049.
37. **Huang, F. Y., A. O. Chan, A. Rashid, D. K. Wong, C. H. Cho, and M. F. Yuen.** 2012. Helicobacter pylori induces promoter methylation of E-cadherin via interleukin-1beta activation of nitric oxide production in gastric cancer cells. Cancer.
38. **Hung, K. H., J. J. Wu, H. B. Yang, L. J. Su, and B. S. Sheu.** 2009. Host Wnt/beta-catenin pathway triggered by Helicobacter pylori correlates with regression of gastric intestinal metaplasia after H. pylori eradication. J Med Microbiol **58:**567-576.
39. **Inoshima, I., N. Inoshima, G. A. Wilke, M. E. Powers, K. M. Frank, Y. Wang, and J. Bubeck Wardenburg.** 2011. A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nat Med **17:**1310-1314.
40. **Johnson, L. G.** 2005. Applications of imaging techniques to studies of epithelial tight junctions. Advanced drug delivery reviews **57:**111-121.
41. **Katz, J., V. Sambandam, J. H. Wu, S. M. Michalek, and D. F. Balkovetz.** 2000. Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. Infect Immun **68:**1441-1449.
42. **Kaurah, P., A. MacMillan, N. Boyd, J. Senz, A. De Luca, N. Chun, G. Suriano, S. Zaor, L. Van Manen, C. Gilpin, S. Nikkel, M. Connolly-Wilson, S. Weissman, W. S. Rubinstein, C. Sebold, R. Greenstein, J. Stroop, D. Yim, B. Panzini, W. McKinnon, M. Greenblatt, D. Wirtzfeld, D. Fontaine, D. Coit, S. Yoon, D. Chung, G. Lauwers, A. Pizzuti, C. Vaccaro, M. A. Redal, C. Oliveira, M. Tischkowitz, S. Olschwang, S. Gallinger, H. Lynch, J. Green, J. Ford, P. Pharoah, B. Fernandez, and D. Huntsman.** 2007. Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. JAMA **297:**2360-2372.
43. **Kirikoshi, H., H. Sekihara, and M. Katoh.** 2001. Up-regulation of WNT10A by tumor necrosis factor alpha and Helicobacter pylori in gastric cancer. Int J Oncol **19:**533-536.
44. **Krueger, S., T. Hundertmark, D. Kuester, T. Kalinski, U. Peitz, and A. Roessner.** 2007. Helicobacter pylori alters the distribution of ZO-1 and p120ctn in primary human gastric epithelial cells. Pathol Res Pract **203:**433-444.
45. **Kurashima, Y., N. Murata-Kamiya, K. Kikuchi, H. Higashi, T. Azuma, S. Kondo, and M. Hatakeyama.** 2008. Deregulation of beta-catenin signal by Helicobacter pylori CagA requires the CagA-multimerization sequence. Int J Cancer **122:**823-831.
46. **Lapointe, T. K., P. M. O'Connor, N. L. Jones, D. Menard, and A. G. Buret.** 2010. Interleukin-1 receptor phosphorylation activates Rho kinase to disrupt human gastric tight junctional claudin-4 during Helicobacter pylori infection. Cell Microbiol **12:**692-703.
47. **Lecuit, M., R. Hurme, J. Pizarro-Cerda, H. Ohayon, B. Geiger, and P. Cossart.** 2000. A role for alpha-and beta-catenins in bacterial uptake. Proc Natl Acad Sci U S A **97:**10008-10013.

48. **Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet, and P. Cossart.** 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science **292:**1722-1725.
49. **Leung, W. K., E. P. Man, J. Yu, M. Y. Go, K. F. To, Y. Yamaoka, V. Y. Cheng, E. K. Ng, and J. J. Sung.** 2006. Effects of Helicobacter pylori eradication on methylation status of E-cadherin gene in noncancerous stomach. Clin Cancer Res **12:**3216-3221.
50. **Machado, J. C., C. Figueiredo, P. Canedo, P. Pharoah, R. Carvalho, S. Nabais, C. Castro Alves, M. L. Campos, L. J. Van Doorn, C. Caldas, R. Seruca, F. Carneiro, and M. Sobrinho-Simoes.** 2003. A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology **125:**364-371.
51. **Marchenko, G. N., N. D. Marchenko, J. Leng, and A. Y. Strongin.** 2002. Promoter characterization of the novel human matrix metalloproteinase-26 gene: regulation by the T-cell factor-4 implies specific expression of the gene in cancer cells of epithelial origin. Biochem J **363:**253-262.
52. **Mateus, A. R., R. Seruca, J. C. Machado, G. Keller, M. J. Oliveira, G. Suriano, and B. Luber.** 2007. EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells. Hum Mol Genet **16:**1639-1647.
53. **Matsuuchi, L., and C. C. Naus.** 2012. Gap Junction Proteins on the Move: Connexins, the Cytoskeleton and Migration. Biochim Biophys Acta.
54. **Matter, K., and M. S. Balda.** 2003. Functional analysis of tight junctions. Methods **30:**228-234.
55. **Mengaud, J., H. Ohayon, P. Gounon, R. M. Mege, and P. Cossart.** 1996. E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell **84:**923-932.
56. **Miehlke, S., C. Kirsch, K. Agha-Amiri, T. Gunther, N. Lehn, P. Malfertheiner, M. Stolte, G. Ehninger, and E. Bayerdorffer.** 2000. The Helicobacter pylori vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. Int J Cancer **87:**322-327.
57. **Moens, E., and M. Veldhoen.** 2012. Epithelial barrier biology: good fences make good neighbours. Immunology **135:**1-8.
58. **Montecucco, C., and R. Rappuoli.** 2001. Living dangerously: how Helicobacter pylori survives in the human stomach. Nat Rev Mol Cell Biol **2:**457-466.
59. **Murata-Kamiya, N., Y. Kurashima, Y. Teishikata, Y. Yamahashi, Y. Saito, H. Higashi, H. Aburatani, T. Akiyama, R. M. Peek, Jr., T. Azuma, and M. Hatakeyama.** 2007. Helicobacter pylori CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. Oncogene **26:**4617-4626.
60. **Nagy, T. A., L. E. Wroblewski, D. Wang, M. B. Piazuelo, A. Delgado, J. Romero-Gallo, J. Noto, D. A. Israel, S. R. Ogden, P. Correa, T. L. Cover, and R. M. Peek, Jr.** 2011. beta-Catenin and p120 Mediate PPARdelta-Dependent Proliferation Induced by Helicobacter pylori in Human and Rodent Epithelia. Gastroenterology **141:**553-564.
61. **Nakayama, M., J. Hisatsune, E. Yamasaki, H. Isomoto, H. Kurazono, M. Hatakeyama, T. Azuma, Y. Yamaoka, K. Yahiro, J. Moss, and T. Hirayama.** 2009. Helicobacter pylori VacA-induced inhibition of GSK3 through the PI3K/Akt signaling pathway. J Biol Chem **284:**1612-1619.
62. **Necchi, V., M. E. Candusso, F. Tava, O. Luinetti, U. Ventura, R. Fiocca, V. Ricci, and E. Solcia.** 2007. Intracellular, intercellular, and stromal invasion of gastric mucosa, preneoplastic lesions, and cancer by Helicobacter pylori. Gastroenterology **132:**1009-1023.
63. **Noach, L. A., T. M. Rolf, and G. N. Tytgat.** 1994. Electron microscopic study of association between Helicobacter pylori and gastric and duodenal mucosa. J Clin Pathol **47:**699-704.

64. **O'Connor, P. M., T. K. Lapointe, S. Jackson, P. L. Beck, N. L. Jones, and A. G. Buret.** 2010. Helicobacter Pylori Activates Calpain Via Toll-Like Receptor 2 to Disrupt Adherens Junctions in Human Gastric Epithelial Cells. Infect Immun.
65. **Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas.** 2000. Translocation of Helicobacter pylori CagA into gastric epithelial cells by type IV secretion. Science **287:**1497-1500.
66. **Odland, G. F.** 1958. The fine structure of the interrelationship of cells in the human epidermis. The Journal of biophysical and biochemical cytology **4:**529-538.
67. **Ogden, S. R., L. E. Wroblewski, C. Weydig, J. Romero-Gallo, D. P. O'Brien, D. A. Israel, U. S. Krishna, B. Fingleton, A. B. Reynolds, S. Wessler, and R. M. Peek, Jr.** 2008. p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. Mol Biol Cell **19:**4110-4121.
68. **Oliveira, M. J., A. C. Costa, A. M. Costa, L. Henriques, G. Suriano, J. C. Atherton, J. C. Machado, F. Carneiro, R. Seruca, M. Mareel, A. Leroy, and C. Figueiredo.** 2006. Helicobacter pylori induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner. J Biol Chem **281:**34888-34896.
69. **Oliveira, M. J., A. M. Costa, A. C. Costa, R. M. Ferreira, P. Sampaio, J. C. Machado, R. Seruca, M. Mareel, and C. Figueiredo.** 2009. CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses Helicobacter pylori-induced cell-invasive phenotype. J Infect Dis **200:**745-755.
70. **Peek, R. M., Jr., G. G. Miller, K. T. Tham, G. I. Perez-Perez, X. Zhao, J. C. Atherton, and M. J. Blaser.** 1995. Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. Lab Invest **73:**760-770.
71. **Perri, F., R. Cotugno, A. Piepoli, A. Merla, M. Quitadamo, A. Gentile, A. Pilotto, V. Annese, and A. Andriulli.** 2007. Aberrant DNA methylation in non-neoplastic gastric mucosa of H. Pylori infected patients and effect of eradication. Am J Gastroenterol **102:**1361-1371.
72. **Petersen, A. M., V. Fussing, H. Colding, J. Blom, A. Norgaard, L. P. Andersen, and K. A. Krogfelt.** 2000. Phenotypic and genotypic characterization of Helicobacter pylori from patients with and without peptic ulcer disease. Scand J Gastroenterol **35:**359-367.
73. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biol **5:**e64.
74. **Polakis, P.** 2000. Wnt signaling and cancer. Genes Dev **14:**1837-1851.
75. **Qian, X., C. Huang, C. H. Cho, W. M. Hui, A. Rashid, and A. O. Chan.** 2008. E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. Cancer Lett **263:**107-113.
76. **Reshetnikova, G., S. Troyanovsky, and D. L. Rimm.** 2007. Definition of a direct extracellular interaction between Met and E-cadherin. Cell Biol Int **31:**366-373.
77. **Reynolds, A. B., and A. Roczniak-Ferguson.** 2004. Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene **23:**7947-7956.
78. **Roczniak-Ferguson, A., and A. B. Reynolds.** 2003. Regulation of p120-catenin nucleocytoplasmic shuttling activity. J Cell Sci **116:**4201-4212.
79. **Schirrmeister, W., T. Gnad, T. Wex, S. Higashiyama, C. Wolke, M. Naumann, and U. Lendeckel.** 2009. Ectodomain shedding of E-cadherin and c-Met is induced by Helicobacter pylori infection. Exp Cell Res **315:**3500-3508.
80. **Segal, E. D., J. Cha, J. Lo, S. Falkow, and L. S. Tompkins.** 1999. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by Helicobacter pylori. Proc Natl Acad Sci U S A **96:**14559-14564.
81. **Selbach, M., S. Moese, C. R. Hauck, T. F. Meyer, and S. Backert.** 2002. Src is the kinase of the Helicobacter pylori CagA protein in vitro and in vivo. J Biol Chem **277:**6775-6778.

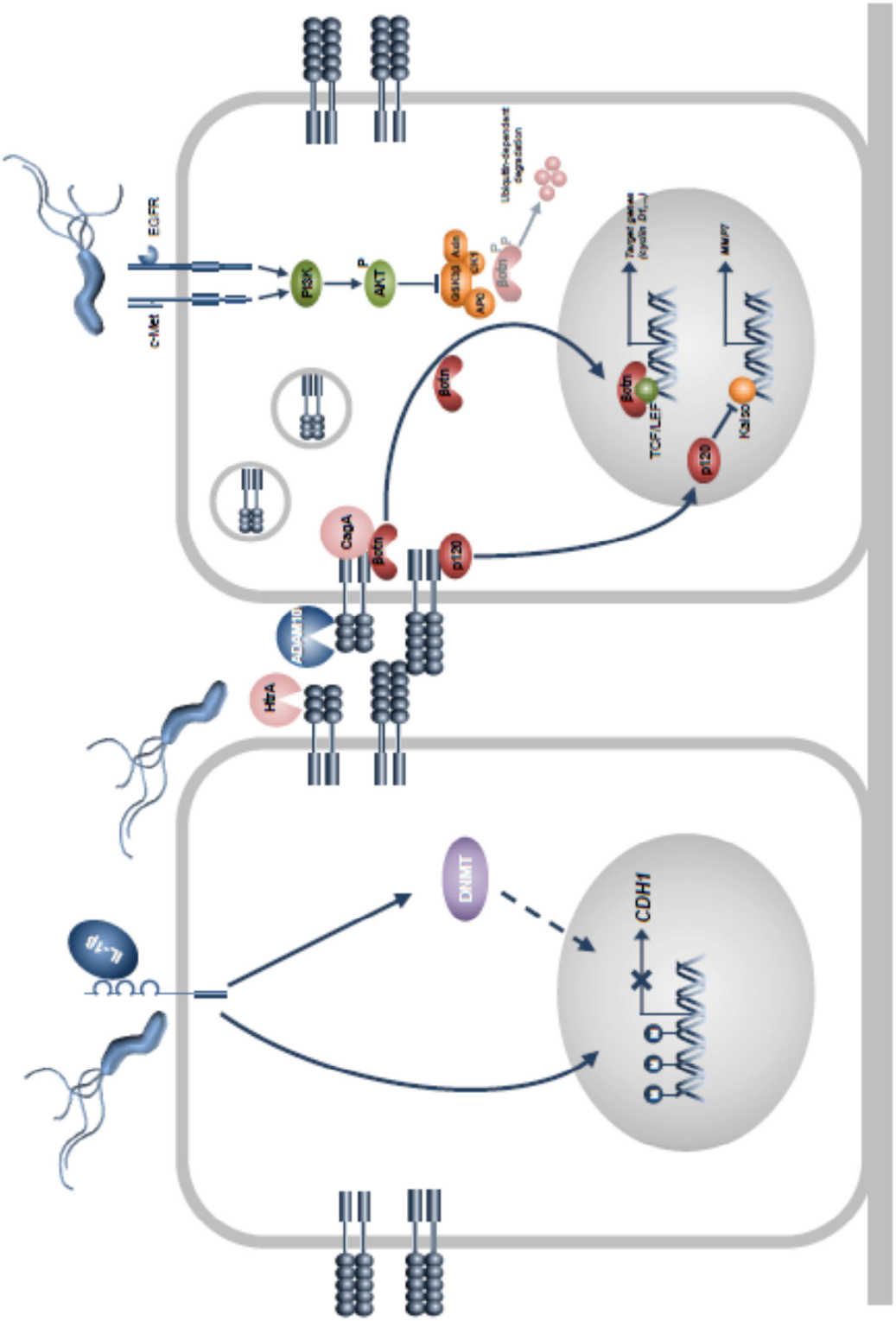
82. **Sokolova, O., P. M. Bozko, and M. Naumann.** 2008. Helicobacter pylori suppresses glycogen synthase kinase 3beta to promote beta-catenin activity. J Biol Chem **283:**29367-29374.
83. **Steck, N., M. Hoffmann, I. G. Sava, S. C. Kim, H. Hahne, S. L. Tonkonogy, K. Mair, D. Krueger, M. Pruteanu, F. Shanahan, R. Vogelmann, M. Schemann, B. Kuster, R. B. Sartor, and D. Haller.** 2011. Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. Gastroenterology **141:**959-971.
84. **Su, B., S. Johansson, M. Fallman, M. Patarroyo, M. Granstrom, and S. Normark.** 1999. Signal transduction-mediated adherence and entry of Helicobacter pylori into cultured cells. Gastroenterology **117:**595-604.
85. **Suerbaum, S., and P. Michetti.** 2002. Helicobacter pylori infection. N Engl J Med **347:**1175-1186.
86. **Sugawara, Y., T. Matsumura, Y. Takegahara, Y. Jin, Y. Tsukasaki, M. Takeichi, and Y. Fujinaga.** 2010. Botulinum hemagglutinin disrupts the intercellular epithelial barrier by directly binding E-cadherin. J Cell Biol **189:**691-700.
87. **Suzuki, M., H. Mimuro, K. Kiga, M. Fukumatsu, N. Ishijima, H. Morikawa, S. Nagai, S. Koyasu, R. H. Gilman, D. Kersulyte, D. E. Berg, and C. Sasakawa.** 2009. Helicobacter pylori CagA phosphorylation-independent function in epithelial proliferation and inflammation. Cell Host Microbe **5:**23-34.
88. **Suzuki, M., H. Mimuro, T. Suzuki, M. Park, T. Yamamoto, and C. Sasakawa.** 2005. Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion. J Exp Med **202:**1235-1247.
89. **Tabassam, F. H., D. Y. Graham, and Y. Yamaoka.** 2009. Helicobacter pylori activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. Cell Microbiol **11:**70-82.
90. **Tammer, I., S. Brandt, R. Hartig, W. Konig, and S. Backert.** 2007. Activation of Abl by Helicobacter pylori: a novel kinase for CagA and crucial mediator of host cell scattering. Gastroenterology **132:**1309-1319.
91. **Tan, S., L. S. Tompkins, and M. R. Amieva.** 2009. Helicobacter pylori usurps cell polarity to turn the cell surface into a replicative niche. PLoS Pathog **5:**e1000407.
92. **Tsukita, S., M. Furuse, and M. Itoh.** 2001. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol **2:**285-293.
93. **Turnberg, L. A.** 1985. Gastric mucosal defence mechanisms. Scand J Gastroenterol Suppl **110:**37-40.
94. **van Hengel, J., P. Vanhoenacker, K. Staes, and F. van Roy.** 1999. Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression. Proc Natl Acad Sci U S A **96:**7980-7985.
95. **Viala, J., C. Chaput, I. G. Boneca, A. Cardona, S. E. Girardin, A. P. Moran, R. Athman, S. Memet, M. R. Huerre, A. J. Coyle, P. S. DiStefano, P. J. Sansonetti, A. Labigne, J. Bertin, D. J. Philpott, and R. L. Ferrero.** 2004. Nod1 responds to peptidoglycan delivered by the Helicobacter pylori cag pathogenicity island. Nat Immunol **5:**1166-1174.
96. **Vikstrom, E., L. Bui, P. Konradsson, and K. E. Magnusson.** 2009. The junctional integrity of epithelial cells is modulated by Pseudomonas aeruginosa quorum sensing molecule through phosphorylation-dependent mechanisms. Exp Cell Res **315:**313-326.
97. **Villar, C. C., H. Kashleva, C. J. Nobile, A. P. Mitchell, and A. Dongari-Bagtzoglou.** 2007. Mucosal tissue invasion by Candida albicans is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. Infect Immun **75:**2126-2135.

98. **Weydig, C., A. Starzinski-Powitz, G. Carra, J. Lower, and S. Wessler.** 2007. CagA-independent disruption of adherence junction complexes involves E-cadherin shedding and implies multiple steps in Helicobacter pylori pathogenicity. Exp Cell Res **313:**3459-3471.
99. **Wilkinson, S. M., J. R. Uhl, B. C. Kline, and F. R. Cockerill, 3rd.** 1998. Assessment of invasion frequencies of cultured HEp-2 cells by clinical isolates of Helicobacter pylori using an acridine orange assay. J Clin Pathol **51:**127-133.
100. **Wroblewski, L. E., L. Shen, S. Ogden, J. Romero-Gallo, L. A. Lapierre, D. A. Israel, J. R. Turner, and R. M. Peek, Jr.** 2009. Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. Gastroenterology **136:**236-246.
101. **Wu, S., K. C. Lim, J. Huang, R. F. Saidi, and C. L. Sears.** 1998. Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. Proc Natl Acad Sci U S A **95:**14979-14984.
102. **Wu, S., P. J. Morin, D. Maouyo, and C. L. Sears.** 2003. Bacteroides fragilis enterotoxin induces c-Myc expression and cellular proliferation. Gastroenterology **124:**392-400.
103. **Wu, S., K. J. Rhee, M. Zhang, A. Franco, and C. L. Sears.** 2007. Bacteroides fragilis toxin stimulates intestinal epithelial cell shedding and gamma-secretase-dependent E-cadherin cleavage. J Cell Sci **120:**1944-1952.
104. **Yamaoka, Y.** 2011. Mechanisms of disease: Helicobacter pylori virulence factors. Nat Rev Gastroenterol Hepatol **7:**629-641.
105. **Yan, F., H. Cao, R. Chaturvedi, U. Krishna, S. S. Hobbs, P. J. Dempsey, R. M. Peek, Jr., T. L. Cover, M. K. Washington, K. T. Wilson, and D. B. Polk.** 2009. Epidermal growth factor receptor activation protects gastric epithelial cells from Helicobacter pylori-induced apoptosis. Gastroenterology **136:**1297-1307, e1291-1293.
106. **Yanagisawa, M., and P. Z. Anastasiadis.** 2006. p120 catenin is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness. J Cell Biol **174:**1087-1096.
107. **Yap, A. S., C. M. Niessen, and B. M. Gumbiner.** 1998. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. J Cell Biol **141:**779-789.
108. **Yokozaki, H.** 2000. Molecular characteristics of eight gastric cancer cell lines established in Japan. Pathol Int **50:**767-777.

Table 1. Examples of infectious agents that target adherens junctions as a means to adhere, enter and/or exploit host cell signaling

Species	Virulence factor	Mechanism of action	Reference
Listeria monocytogenes	InlA	Adhesion to E-cadherin and host cell invasion	12
Streptococcus pneumoniae	PsaA	Adhesion to E-cadherin and host colonization	16
Candida albicans	Als3	Adhesion to E-cadherin and host cell invasion	15
	Sap5p	Extracellular and intracellular E-cadherin cleavage	23
Bacteroides fragilis	BFT	Extracellular and intracellular E-cadherin cleavage	17, 18, 19
Porphyromonas gingivalis	HRgpA, RgpB, Kgp	Extracellular E-cadherin cleavage	20
Enterococcus faecalis	GelE	Extracellular E-cadherin cleavage	21
Staphylococcus aureus	Hla	Extracellular E-cadherin cleavage via ADAM-10	24
Clostridium botulinum	BoNT	E-cadherin, disrupting epithelial barrier	25
Pseudomonas aeruginosa	?	Alteration of phosphorylation status and delocalization of the adherens junctions proteins	26

Figure 1


EGFR
c-Met
PI3K
P
AKT
Ubiquitin-dependent degradation
Axin
GSK3
APC
βcatn
TCF/LEF
p120
Kaiso
CagA
ADAM10
HtrA
IL-1β
DNMT
CDH1

Paper IV

***Epithelial E- and P-cadherins: Role and clinical significance in cancer*.**

Paredes J, Figueiredo J, Albergaria A, Oliveira P, Carvalho J, Ribeiro AS, Caldeira J, **Costa AM**, Simões-Correia J, Oliveira MJ, Pinheiro H, Pinho SS, Mateus R, Reis CA, Leite M, Fernandes MS, Schmitt F, Carneiro F, Figueiredo C, Oliveira C, Seruca R.

Biochimica et Biophysica Acta - Reviews on Cancer 2012 May 19; 1826(2):297-311

Biochimica et Biophysica Acta 1826 (2012) 297–311

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbacan

Review

Epithelial E- and P-cadherins: Role and clinical significance in cancer

Joana Paredes [a,b], Joana Figueiredo [a], André Albergaria [a], Patrícia Oliveira [a], Joana Carvalho [a], Ana Sofia Ribeiro [a], Joana Caldeira [a,c], Ângela Margarida Costa [a], Joana Simões-Correia [a], Maria José Oliveira [c], Hugo Pinheiro [a], Salomé S. Pinho [a,d], Rita Mateus [a], Celso A. Reis [a,b,d], Marina Leite [a], Maria Sofia Fernandes [a], Fernando Schmitt [a,b], Fátima Carneiro [a,b], Céu Figueiredo [a,b], Carla Oliveira [a,b], Raquel Seruca [a,b,*]

[a] IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Porto, Portugal
[b] Medical Faculty of the University of Porto, Porto, Portugal
[c] NewTherapies Group, INEB-Institute for Biomedical Engineering, Porto, Portugal
[d] Instituto de Ciência Biomédicas Abel Salazar (ICBAS), Porto, Portugal

ARTICLE INFO

Article history:
Received 18 January 2012
Received in revised form 9 May 2012
Accepted 11 May 2012
Available online 19 May 2012

Keywords:
E-cadherin
P-cadherin
Cancer
Gastric
Breast
Hereditary diffuse gastric cancer

ABSTRACT

E-cadherin and P-cadherin are major contributors to cell–cell adhesion in epithelial tissues, playing pivotal roles in important morphogenetic and differentiation processes during development, and in maintaining integrity and homeostasis in adult tissues. It is now generally accepted that alterations in these two molecules are observed during tumour progression of most carcinomas. Genetic or epigenetic alterations in E- and P-cadherin-encoding genes (*CDH1* and *CDH3*, respectively), or alterations in their proteins expression, often result in tissue disorder, cellular de-differentiation, increased invasiveness of tumour cells and ultimately in metastasis. In this review, we will discuss the major properties of E- and P-cadherin molecules, its regulation in normal tissue, and their alterations and role in cancer, with a specific focus on gastric and breast cancer models.

Contents

* Corresponding author at: IPATIMUP, Institute of Molecular Pathology and Immunology of the University of Porto, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal. Tel.: +351 225570700; fax: +351 225570799.
E-mail address: rseruca@ipatimup.pt (R. Seruca).

0304-419X/$ – see front matter
doi:10.1016/j.bbcan.2012.05.002

1. Introduction

Cadherin superfamily is mainly composed by 1) classical cadherins, which are the major components of cell–cell adhesive junctions; 2) non-classical cadherins, which include desmosomal cadherins; and 3) protocadherins, which are implicated in neuronal plasticity [1,2]. E- and P-cadherins belong to the subfamily of classical/type I cadherins that comprises only four members: the non-neuronal epithelial (E-) and placental (P-) cadherins, and the neuronal neural (N-) and retinal (R-) cadherins [3].

The epithelial-calcium dependent cell–cell adhesion is achieved by the establishment of homophilic interactions between two cadherin molecules of adjacent cells to form a homodimer [4,5]. E- and P-cadherin mature proteins are organised in three major structural domains: a large extracellular domain, a single membrane-spanning segment and a short cytoplasmic domain. The extracellular domains are composed by 5 cadherin repeats (EC), which constitute a key element for their classification. These extracellular domains are sequences of 110 residues, which are present only in cadherins, commonly designated as EC1–EC5 [6]. The EC1 domain is the main responsible for cadherins adhesive properties, which are organised between cells in a zipper-like structure, a trademark of these proteins [7]. The normal conformation of E- and P-cadherin molecules is only stable in the presence of Ca^{2+} in the surrounding microenvironment, whose binding with the extracellular portion of the polypeptide chain is a prerequisite for cadherin mediated cell–cell adhesion. Calcium-binding sites consist of short highly conserved aminoacid sequences that are located between neighbouring EC repeats [8]. E- and P-cadherin highly conserved cytoplasmic domain (ICD) consists of approximately 150 aminoacids [9], interacting with β-, α-, and γ (plakoglobin)-catenins (βctn, αctn, γctn) [8,10], forming a complex, and being linked to the actin cytoskeleton through αctn [11]. Later, the binding to p120-catenin (p120ctn) stabilises cadherins at the cell membrane and promotes its correct function [12]. In Fig. 1, the structure of human E- and P-cadherin gene and protein is represented, as well as the link with the actin cytoskeleton through catenins, establishing the cadherin–catenin complex. The stability of this complex is required for providing normal cell–cell adhesion and homeostatic tissue architecture. Further, there is evidence that alterations in the adhesion properties between cells endow them with an invasive and migratory phenotype. Indeed, changes in the expression or function of cell–cell adhesion molecules, such as E-and P-cadherin, have been implicated in all steps of tumour progression, including detachment of tumour cells from the primary site, intravasation into the blood stream, extravasation into distant target organs, and formation of secondary lesions or metastasis [13–15]. In this review, as first topic, we describe the role of both molecules in normal tissue homeostasis; however, alterations of E- and P-cadherin associated to cancer are the major focus of this report.

2. E- and P-cadherin: gene structure, regulation and function

2.1. Gene structure

The genes encoding E- and P-cadherin, *CDH1* and *CDH3*, are both annotated to the human chromosome 16q22.1, being *CDH3* 32 kb upstream of *CDH1* (Fig. 1A) [16]. Both genes harbour a 5′-located CpG island in their promoters [17,18]. The transcription start site (TSS) of *CDH1* is currently annotated at the coordinate 68,771,128 bp, whereas the translation start site (ATG) is 194 bp downstream [19]. The canonical promoter of *CDH1* starts at least 125 bp upstream of the TSS and ends 27 bp downstream of it [20,21]. This area does not have a TATA box, but includes several regulatory elements such as GC-boxes, E-boxes (or E-pal box) and a CCAAT-box, all highly conserved across mammals [21,22]. Interestingly, an Alu repeat (*AluJo*), that may uncover putative new molecular mechanisms of gene regulation, is found less than 500 bp upstream from the canonical ATG. Concerning *CDH3* gene, its TSS is currently annotated to the coordinate 68,678,739 bp on the forward strand and the ATG is found 553 bp downstream of the TSS. The *CDH3* promoter exhibits no TATA box, as well as *CDH1*, but includes a CAAT box, two putative AP2-binding

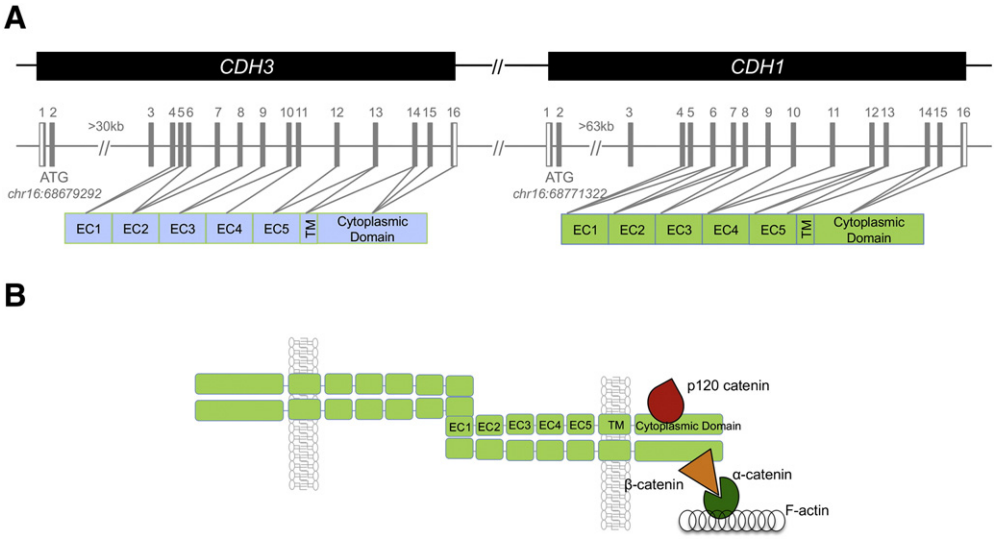


Fig. 1. Structure of the human CDH3 and CDH1 genes and the encoded P- and E-cadherin proteins. (A) The CDH3 and CDH1 genes are located on chromosome 16q22.1 and exhibit structural similarities: both display an equal exon number (16) and a large intron after exon 2. P- and E-cadherin mature proteins are organised in three major structural domains: a large extracellular domain composed by 5 cadherin repeats (EC), a single membrane-spanning segment (TM) and a short cytoplasmic domain. (B) The cytoplasmic domain of cadherins interacts with actin cytoskeleton through a complex of proteins including p120-, β- and α-catenins.

motifs and a GC-rich region containing putative Sp1-binding sites, all of which highly conserved within the promoter sequence of E-cadherin [20]. However, no sequence homologous to the palindromic sequence E-pal, observed on the E-cadherin promoter, has been found in the 5′ region of the P-cadherin gene [20]. Similarly to *CDH1*, an *AluJb* repeat is found ~700 bp upstream of the ATG, putatively enclosing gene regulation or exonisation features [20,23].

Concerning the non-coding part of the *CDH1* and *CDH3* genes, the intron 2 in both genes has more than 63 Kb and 30 kb in length, respectively [24]. This large intron is a structurally conserved feature across mammals, which may suggest the presence of common *cis*-regulatory elements, yet to be described. In addition, EST and CAGE annotations have shown interesting data, supporting new areas of transcription within this large intron of the *CDH1* gene [24–26]. As *CDH1*, an interesting feature of *CDH3* intron 2 is the presence of a promoter associated regulatory feature, recalling the insulator element. In addition, several EST and CAGE tags were also annotated within this gene [19,25].

Ensembl database currently describes 4 transcripts arising from the *CDH1* gene locus [24]. Concerning *CDH3*, two transcripts are currently annotated at its gene locus. In conclusion, *CDH1* and *CDH3* genes share a common genetic structure with several conserved features and regulatory elements, yet to be described, within their non-coding sequences.

2.2. Transcriptional regulation

The control of E-cadherin epithelium-specific expression results from the combined interplay of various proteins and regulatory elements. Through the binding sites located at *CDH1* 5′ sequence, Retinoblastoma (Rb), c-Myc and WT1 promote *CDH1* activation [27]. Another positive *CDH1* regulator is the hepatocyte nuclear factor-3 (HNF3) that synergises with AML-1 and p300 to stimulate E-cadherin transcription [28]. At the same time, *CDH1* can be negatively regulated by the inactivation of the E-pal box, containing two adjacent E-boxes in the promoter [29]. SNAIL was the first identified E-cadherin-specific transcriptional repressor interacting with this E-pal element [30]. Slug (also known as SNAI2) and E12/E47 were afterwards confirmed as potent negative regulators of E-cadherin expression [31,32]. ZEB1 and ZEB2 have also emerged as key factors negatively regulating E-cadherin [33,34]. These transcriptional repressors exert their action by recruiting transcriptional corepressors, such as CtBP and mSinA [35,36]. The transcriptional activity of E-cadherin promoter can be repressed by the combined action of the β-ctn-Lef1/TCF complex, in a Lef1/Tcf binding site dependent manner [37]. Several other factors have been described to act upon E-cadherin gene, but not in such a specific way, targeting multiple adhesion and polarity genes during developmental epithelial to mesenchymal transition (EMT), like Twist1, HOXB7, CBF-A (HNRPAB) and KLF8 [38–40]. Hypermethylation at the *CDH1* promoter CpG island is an important mechanism to control the expression of the gene and, while normal epithelial cells carry unmethylated *CDH1* promoter, the opposite occurs in many types of cancers, as has been first described by Graff and collaborators in 1995 [41].

Data concerning *CDH3* promoter regulation by transcription factors is still very limited. Recently, it was demonstrated that *CDH3* gene is a direct transcriptional target of p63 [42], playing an import role in the regulation of expression programs involved in cell–cell adhesion [43]. In addition, it was shown that β-ctn is also associated with *CDH3* promoter and activates its expression, but in a LEF/TCF independent specific-manner. In fact, it was shown that activation of β-ctn signalling correlates with up-regulation of P-cadherin expression, as well as downregulation of endogenous β-ctn levels, by RNA interference, inhibited P-cadherin promoter activity [44]. Concerning *CDH3* repression, we verified that methylation of its gene promoter was associated with negative P-cadherin expression in normal epithelial cells [23].

2.3. Post-translational regulation

E-cadherin post-translational regulation mechanisms have been extensively studied, but the same holds not true for P-cadherin. Intracellular trafficking is an important mean of regulating the levels of E-cadherin at the cell surface (Fig. 2) [45,46].

The exocytic traffic controls the delivery of newly synthesised E-cadherin to the plasma membrane through recycling endosomes. During this process, β-ctn binds to E-cadherin and these two proteins are transported to the plasma membrane as a complex [47,48]. Some endosome proteins, as Rab11 and Sorting Nexin 1 (SNX1), have also been implicated in cadherin recycling [49,50]. In fact, depletion of cellular SNX1 levels by siRNA or inhibitory mutants of Rab11 block normal E-cadherin trafficking to the cell surface [49,50]. Recently, type Iγ phosphatidylinositol phosphate kinase (PIPKIγ) was found to bind directly to E-cadherin, modulating its cellular trafficking. Disruption of PIPKIγ binding to E-cadherin, results in defects in E-cadherin transport and blocks adherens junctions assembly [51].

Additionally, the endocytic pathway also controls the internalisation of E-cadherin with consequences on protein turnover, recycling, sequestration and degradation [46,52]. p120ctn is the best characterised inhibitor of E-cadherin endocytosis, by hampering the binding of adaptor complexes that would recruit the clathrin-coated pits, and by stabilising cadherins at the cell surface [53–55]. In contrast, although p120ctn also binds to P-cadherin [53,54], it is known that the overexpression of this cadherin is strongly associated with cytoplasmic accumulation of p120ctn, since it competes for its binding with endogenous E-cadherin [56] (and unpublished results). Interestingly, when the specific binding site of p120ctn at the juxtamembrane domain (JMD) of P-cadherin is completely absent or mutated, P-cadherin is not functional as a pro-invasive molecule [57].

Another key modulator of E-cadherin movement, along with the endocytic pathway, is the ADP-ribosylation factor 6 (ARF6) [58,59]. ARF6 activation results in the internalisation of E-cadherin from cell–cell junctions into early endosomes. ARF6-GTP interacts and recruits Nm23-H1 from the cytosol to adherens junctions, leading to endosomal dependent E-cadherin sequestration [60]. The recruitment of Nm23-H1 has also been coupled to downregulation of Rac1 activity, a process that facilitates disassembly of adherens junctions [61]. Recently, it was demonstrated that EGFR interacts specifically with an ARF6 guanine nucleoside-exchange factor (GEP100/BRAG2), promoting ARF6 activation and consequent E-cadherin internalisation [62].

Tyrosine kinases, such as Src or other tyrosine kinase receptor (RTKs), also regulate the expression, function and trafficking of E-cadherin [63]. Tyrosine phosphorylation of E-cadherin–catenin complex results in E-cadherin endocytosis and disturbance of the adhesion complex. Src activation induces EMT, targeting E-cadherin to ubiquitination and then shuttled to the lysosome, instead of following its normal trafficking route of recycling back to the lateral plasma membrane [64]. Furthermore, the expression of v-Src also induces the activation of the GTPases Rab5 and Rab7, and expression of the dominant-negative mutants of these GTPases introduces stage-specific blocks in the trafficking of E-cadherin to the lysosome [64]. Ubiquitination of E-cadherin is an additional level of protein regulation and is mediated by Hakai, a c-Cbl-like E3 ubiquitin ligase. Hakai induces the mono-ubiquitination of E-cadherin in response to Src activation resulting in E-cadherin lysosomal degradation [65].

Another important level of post-translational regulation of E-cadherin is by glycosylation. E-cadherin can be post-translationally modified by *O*- and *N*-glycosylation, which modifications have been

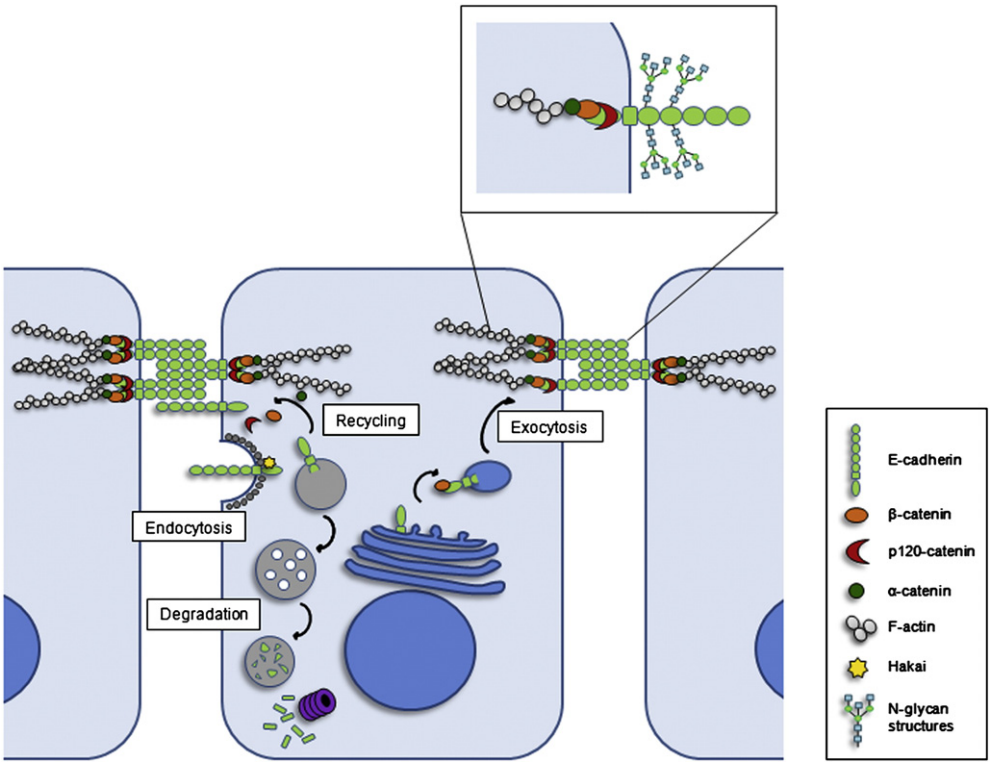


Fig. 2. Illustration of classical cadherin trafficking and post-translational regulation. Newly synthesised cadherins are trafficked from the trans-Golgi network to the plasma membrane in association with β-catenin. At the plasma membrane, p120-catenin binds to the cadherin juxtamembrane domain, stabilising and preventing the entry of cadherin into degradative endocytic pathways. p120-deprived cadherin is prone to interact with other proteins, such as clathrin adaptor proteins and Hakai, promoting E-cadherin internalisation by clathrin-dependent pathways. After internalisation, E-cadherin can be recycled back to the plasma membrane or targeted for degradation. The box in the upper figure represents the post-translational modification of E-cadherin by N-glycosylation. E-cadherin has four potential N-glycosylation sites at the extracellular domain and the N-glycans are crucial for E-cadherin stability at plasma membrane and function.

described to have an effect on E-cadherin functionality [66]. Cytoplasmic O-glycosylation (O-GlcNAc) of newly synthesised E-cadherin was described to block its cell surface transport, resulting in reduced intercellular adhesion [67]. Furthermore, *N*-glycans seem to be essential for E-cadherin expression, folding and trafficking [68,69]. The extracellular domain of human E-cadherin has four potential *N*-glycosylation sites on the basis of amino acid sequence (Fig. 2) and those *N*-glycans represent the most prominent modification of the molecule, contributing up to 20% of its total mass [69]. Recently, we demonstrated that N-acetylglucosaminyltransferase III and V (GnT-III and GnT-V) competitively modified E-cadherin N-glycans, modifying its membranous expression [70]. GnT-III knockdown cells revealed a membrane de-localisation of E-cadherin leading to its cytoplasmic accumulation. Further, GnT-III knockdown also caused modifications of E-cadherin N-glycans catalysed by GnT-III and GnT-V. Altogether, we verified the existence of a bidirectional crosstalk between E-cadherin and GnT-III/GnT-V contributing to E-cadherin post-translational regulation [66,70]. Recently, we identified for the first time, the role of GnT-III-mediated glycosylation of E-cadherin as a novel and major component of EMT, but also of mesenchymal to epithelial transition (MET) signatures, using the spontaneously immortalised normal mammary epithelial cell line *EpH-4* [71]. Moreover, the core fucosylation of E-cadherin was also described to have a role in the regulation of cell–cell adhesion in cancer [72].

2.4. Protein function

The first description on E-cadherin's cell-to-cell adhesive properties was reported in the V79 Chinese hamster lung cell line, by Takeichi in 1977 [73]. E- and P-cadherin are indeed the first adhesion molecules that are expressed in the mouse embryo. The proper development of an embryo is assured under conditions of regulated cell–cell and cell–matrix adhesion. At one cell stage of embryogenesis, uvomorulin, the original name of E-cadherin, has been shown to be present [74–76]. At the 8-cell stage, E-cadherin is essential for the compaction of the morula and the subsequent organisation of epithelial tissues [77]. Using mouse models, it was clearly shown that embryo implantation into the uterine epithelium involves both E- and P-cadherin. In fetal development, E-cadherin was found to be expressed only in the embryonic region of placenta with a sharp boundary to the maternal region [76,78,79]. In contrast, P-cadherin expression was observed in the placenta, both in the embryonic and maternal regions, hence the classical denomination of placental-cadherin. These observations suggest complementary roles of the two cadherins during development. E-cadherin is essential in preventing the embryonic tissues from mixing with the maternal tissues, while P-cadherin is required for association of embryonic and maternal tissues.

The importance of E- and P-cadherin to normal development is well demonstrated by gene knockouts (KO) in mice. E-cadherin KO is lethal at very early stages in embryogenesis, due to the failure of trophoectoderm formation, the first polarised epithelial layer in the mouse embryo [80,81]. In contrast, germline loss of function of P-cadherin is not lethal, but is associated to developmental defects namely of the breast. Interestingly, it was verified that virgin P-cadherin null female mice exhibit precocious differentiation of the mammary gland, indicating that P-cadherin mediated adhesion or signals derived from its cell–cell interactions are indeed important determinants of mammary gland growth control and in the maintenance of an undifferentiated state during a specific period of time [82].

Germline mutations of E-cadherin and P-cadherin are associated to human developmental defects. In the case of E-cadherin, germline mutations of E-cadherin, leading to various aberrant transcripts, were

associated to congenital midline malformations, namely for the craniofacial morphogenesis. In fact, specific alterations of E-cadherin pathway during development maybe involved in the genesis of midline congenital malformations, such as lip and palate clefting [83]. P-cadherin germline mutations have been associated to: 1) hypotrichosis with juvenile macular dystrophy (HJMD) and 2) ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM syndrome) [42,84,85].

In adult tissues, E-cadherin plays a major role in the formation of epithelia, maintenance and homeostasis. Its function lies primarily in the formation of adherens junctions, which are crucial for the initiation and maintenance of a homeostatic intercellular space and cell-to-cell interaction in a wide variety of tissues and cell populations [86]. E-cadherin, besides providing epithelial cells with structural integrity also functions as a landmark, spatially confining signalling molecules and polarity cues, as well as serving as docking sites for vesicles [86]. P-cadherin also contributes to cell-to-cell adhesion, but its expression is restricted to specific areas of epithelial tissues (normally proliferating regions, like the germinative layer of the surface epidermis), co-localising partially with E-cadherin expression [87].

3. E-cadherin and cancer

It is well accepted the E-cadherin plays an important role as an invasion suppressor gene/protein, since its loss of expression, abnormal function, or both, leads to an increased ability of cells to invade neighbouring tissues, as verified in cancer [88]. In contrast, P-cadherin expression is correlated with cell dedifferentiation and increased cell proliferation, as well as with the connection or segregation of epithelial cell layers [79,89,90]. Recently, P-cadherin has also been described as an invasion promoting protein in some cancer models [91]. In this chapter, we describe mechanisms of deregulation of E-cadherin in cancer separated from those described for P-cadherin, for the sake of simplicity.

3.1. CDH1 structural alterations

E-cadherin gene (*CDH1*) loss/abnormal expression leads neoplastic cells to invade neighbouring tissues/organs, as shown in several models of human cancer, including carcinomas of the breast and stomach. In breast cancer, downregulation of E-cadherin is a specific feature of lobular carcinomas [92–95], being also observed in pleomorphic lobular carcinomas [96]. Further, its reduced expression can also be associated with some non-lobular breast carcinomas of triple negative phenotype, like the metaplastic carcinomas [97,98]. In lobular breast cancer, somatic truncating mutations of the *CDH1* gene occur throughout its extracellular domain [99]; however, germline *CDH1* mutations were rarely found in invasive lobular breast cancers (LBC) with or without diffuse gastric cancer associated [100,101].

In gastric cancer, downregulation of E-cadherin is a feature of diffuse/isolated cell carcinomas and occurs in the large majority of the cases (Fig. 3) [102–106]. E-cadherin absence or aberrant expression may be caused, in sporadic diffuse/mixed gastric cancer cases, by somatic mutations that cluster in the central region of *CDH1* gene, leading to the skipping of exon 8 or exon 9 [102,103] in frequencies that vary between 20 and 60% of the cases [106,107]. In hereditary forms of diffuse gastric cancer, the well-known hereditary diffuse gastric cancer syndrome (HDGC) described by Guilford in 1998, germline alterations of *CDH1* gene are causing events [108]. Overall, around 45% of HDGC families, selected on the basis of established clinical criteria, harbour *CDH1* germline alterations [109]. So far, over one hundred HDGC and two LBC families have been described to harbour *CDH1* germline alterations [109–111]. The most frequent type of *CDH1* germline alterations are point and small frameshift mutations. In terms of the predicted impact of these germline *CDH1* mutants, 80% result in protein truncation or even complete lack of protein expression. The remaining 20% of *CDH1* mutations are of the missense type and the pathogenic relevance of such sequence *CDH1* variants implicates a series of *in silico* and *in vitro* tests, developed by our group, to functionally characterise their functional impact [112–118]. In 2009, Oliveira et al. described, for the first time, *CDH1* large genomic deletions in apparently mutation negative HDGC families [109]. Regarding structural alterations leading to somatic inactivation of *CDH1* alleles in HDGC tumours, we and others have shown that 20–30% of HDGC primary tumours carry *CDH1* point mutations, intragenic deletions or LOH [119–122]. But importantly, our group has shown that LOH is the most frequent event in lymph node metastases from HDGC patients and is present in as much as 60% of all metastases analysed [121].

3.2. CDH1 transcriptional deregulation

Overexpression of E-cadherin repressors has been described to be present in cancer [123–126]. In 2008, it was shown that *CDH1* transcriptional silencing could be also due to histone H3-K9 deacetylation [127]. Peinado and colleagues demonstrated that Snail represses the expression of E-cadherin through recruitment of a multimolecular complex composed by mSin3A corepressor and HDAC1/2 to E-cadherin promoter. Further, Snail-expressing cells were enriched in deacetylated histones H3 and H4 [123].This link between the HDAC machinery/Snail and silencing of E-cadherin was also corroborated, *in vivo*, in a metastatic pancreatic cancer model [128]. Others have also showed that *CDH1* repression by Snail was dependent on complexes involving histones, namely the activity of the Polycomb repressive complex 2 (PRC2). Snail1 represses E-cadherin expression by recruiting one component

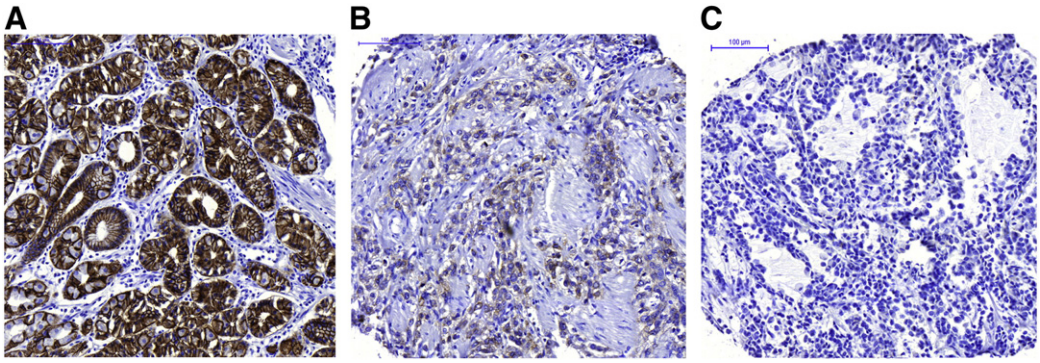


Fig. 3. Immunohistochemistry panel representing the expression pattern of E-cadherin in normal gastric mucosa and in gastric carcinomas in a tissue microarray. (A) In normal gastric mucosa, E-cadherin is expressed at the membrane of epithelial cells (magnification: 200×); Diffuse gastric carcinomas showing reduced and aberrant expression of E-cadherin (B) or complete loss of E-cadherin expression (C) (magnification: 200×).

of PRC2 (Suz12) and, in tumour cell lines, silencing of Suz12 leads to the silencing of E-cadherin expression by trimethylation of lysine 27 on histone H3 [129]. The same mechanism of E-cadherin silencing is verified for another component of PRC2, namely EZH2, which has also been implicated in cancer [124,125]. Increased levels of EZH2 have been observed in aggressive tumours, and treatment of cells with HDAC inhibitors prevents EZH2-mediated downregulation of E-cadherin and reduces cellular invasion [124,125].

MicroRNAs (miRNAs) have also emerged as a new layer of *CDH1* gene regulation [130,131]. Members of the miR-200 family of microRNAs have been implicated in the regulation of E-cadherin expression, through direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2 [132–135]. Inhibition of endogenous miR-200 expression levels lead to a relief of ZEB1 and ZEB2 repression, reduction of E-cadherin mRNA expression levels and, consequently, to a mesenchymal-like morphology and increased cell migration. In human breast cancer tissues, loss of miR-200 family expression was observed in highly aggressive, metaplastic type of breast cancer, lacking E-cadherin expression [134]. A significant correlation between downregulation of miR-200 family members, increased expression of ZEB1, and reduced expression of E-cadherin was observed in primary serous papillary ovarian tumours [135].

In addition to the miR-200 family, miR-9 and miR-101 have also been implicated in the network of E-cadherin regulation [106,136,137]. miR-9 was initially found to be expressed in primary hepatocellular carcinomas (HCC) with metastases when compared with HCC without metastases [136]. Although, E-cadherin was first postulated as a putative target of miR-9, since its expression was increased when cells were treated with a miR-9 inhibitor, the formal proof of miR-9 impact on E-cadherin was only achieved very recently. It was shown that miR-9, induced by Myc in breast cancer cells, directly targets E-cadherin and, subsequently, activates β-ctn signalling and VEGF expression, promoting cell motility, invasion and tumour angiogenesis [137]. Our group have recently uncovered a pathway that causes E-cadherin dysfunction in gastric cancer via loss of miR-101. We found that miR-101 was significantly downregulated in gastric tumours in comparison with normal gastric mucosas and, at least in 65% of the cases analysed, this downregulation was caused by deletions and/or microdeletions at miR-101 genomic loci. Moreover, around 40% of the cases showing miR-101 downregulation, displayed concomitant EZH2 overexpression which, in turn, was associated with loss/aberrant E-cadherin expression, specifically in the intestinal-type gastric cancer [106,137].

Apart from all these modifiers of E-cadherin expression and function, the most widely studied, and probably the most frequent, mechanism in human cancer that leads to *CDH1* downregulation is hypermethylation of its promoter. This mechanism has been described either in epithelial, with greater emphasis on gastric cancer and non-epithelial cancers, such as the case of acute myelogenous leukemia (AML) [107,138]. Specifically in gastric cancer, E-cadherin methylation has been extensively studied by our group. We have reported E-cadherin methylation in frequencies that vary between 6% and 60% of the cases, in different series of sporadic diffuse gastric cancer [106,107] (and unpublished results). Although less frequently studied, cadherin methylation has also been described in sporadic intestinal-type gastric cancer in approximately 15% of the cases [106] (and unpublished results). Additionally, it was also found that the most common mechanism of biallelic inactivation of the *CDH1* gene in HDGC is promoter hypermethylation [117,119–122]. In HDGC primary tumours from *CDH1* germline mutation carriers, CDH1 methylation has been described in 50 to 70% of the cases [117,119–122], either as a single event (~50%) or concomitantly with LOH at the CDH1 locus (~19%) [121]. Interestingly, the frequency of *CDH1* promoter methylation decreases in HDGC lymph node metastasis to ~25%, almost invariably accompanied by LOH in the same lesion. Because of the finding of somatic alteration heterogeneity in HDGC neoplastic lesions, drugs targeting only epigenetic alterations are likely to be effective only in a limited percentage of gastric carcinomas within the setting of HDGC.

3.3. Post-translational E-cadherin deregulation

Recently, E-cadherin trafficking deregulation was shown to be associated to epithelial cancer progression. The importance of E-cadherin trafficking deregulation in cancer has been explored by our group using E-cadherin missense mutations, associated to HDGC, as a model. We have recently shown that two HDGC-associated missense mutations functionally inactive, R749W and E757K, are regulated by Endoplasmic Reticulum Associated Degradation (ERAD), and prematurely degraded by the proteasome [139]. ERAD is the mechanism by which newly synthesised misfolded proteins are translocated from the ER to the cytosol and are degraded by the ubiquitin-proteasome system [140,141]. We proposed that these R749W and E757K HDGC-associated mutations interfere with the folding of E-cadherin and originate misfolded proteins that are recognised by the ERAD machinery, triggering their degradation, and leading consequently to the loss of E-cadherin protein expression and function [139]. Nevertheless, there are other indirect evidences showing the role of E-cadherin trafficking deregulation in cancer progression. One example is proposed by the work of Toyoshima and co-workers who have recently found that Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) is involved in the lysosomal trafficking of E-cadherin and its disruption impacts E-cadherin degradation inhibiting tumorigenesis in nude mice and metastasis formation. Another example of an indirect regulator of E-cadherin is p120ctn, which is an E-cadherin stabiliser. p120 alterations in cancer are frequent [142], including its downregulation, and are correlated with E-cadherin loss or abnormal expression in different cancer types, like in gastric cancer [143–145]. Even so, there are no direct evidences showing that the alterations of p120ctn in cancer lead to E-cadherin trafficking defects in the tumour context and which are the underlying mechanisms, timing and associated functional consequences.

Aberrant glycosylation has been also implicated as an essential mechanism defining stage and fate of tumour progression [146,147] and it has recently been proposed that glycosylation can be a mechanism of E-cadherin deregulation in cancer [66]. It has been shown that E-cadherin displays a modified *N*-glycosylation pattern during the acquisition of the malignant phenotype [148]. These cancer related changes in the pattern of E-cadherin *N*-glycosylation included the synthesis of branched N-glycans (β1,6 GlcNAc branched structures) and an increase in sialylation (α2,3 and α2,6 linked sialic acids). These observations support the role of N-glycans on E-cadherin-mediated tumour progression [148]. The remodelling of E-cadherin *N*-glycans catalysed by GnT-III and GnT-V was also shown to have a potential role on E-cadherin mediated cell–cell adhesion and cancer [149,150]. The glycosylation of E-cadherin by GnT-III seems to participate in the suppression of metastases of melanoma cells [150]. Interestingly, in an *in vitro* and an *in vivo* model of human breast and gastric cancer, we showed that aberrant E-cadherin *N*-glycosylation can be considered as a potential new mechanism of E-cadherin deregulation in cancer, operating at the post-translational level [66,70].

3.4. E-cadherin and signalling pathways

Increasing evidences indicate that E-cadherin mediated carcinogenesis depends on the aberrant activation of signalling pathways and interaction with other molecules (such as cytoskeletal components, integrins, and growth-factor receptors, among others) [1,151–153]. It has been demonstrated that E-cadherin can compete with the βctn/TCF-mediated transcriptional activity of the canonical WNT signalling pathway, increasing in this way proliferation [154,155]. However, E-cadherin loss of function rarely leads to a nuclear accumulation of βctn and increased cell proliferation in primary carcinomas even in cases with E-cadherin mutations (V832M) localised in the βctn binding domain [156]. Another E-cadherin-βctn associated signalling regards regulation of the cytoskeletal network, which organisation is modulated by the activity of the members of the Rho family of small GTPases with

direct consequences in actin filaments [157]. Three members of the Rho small GTPase subfamily are involved: Rho, Rac and Cdc42. Cdc42 induces the formation of filipodia that are important in defining the directionality of the cell movement, Rac induces formation of lamellipodia at the leading edge of migrating cells, and Rho regulates contractile forces to move the body and the tail of a migrating cell behind the leading edge and induces formation of actin stress fibres. E-cadherin in-frame deletions of exon 8 commonly found in sporadic diffuse gastric cancer (HDGC), lead to a reduction in Rac1 activation and to an inhibition of Rho [158]. In Hereditary diffuse gastric carcinoma, E-cadherin germline missense mutations clustering on the extracellular domain induce increased RhoA activity leading to increase of cell migration ability [114,159,160].

Additionally, E-cadherin has been shown to colocalise with several RTKs to basolateral areas of polarised epithelial cells and to form multicomponent complexes with them [161,162]. Numerous studies indicate that several RTKs can downregulate and inhibit E-cadherin-dependent adhesion and induce the morphologic transformation, known as EMT [65,163]. On the other hand, E-cadherin can inhibit the activation and signalling through RTKs, important for cellular homeostasis, implying a bidirectional regulation [164]. In particular, EGFR has been reported to be involved in a bidirectional crosstalk with E-cadherin. EGF-dependent activation of EGFR can be inhibited in an E-cadherin adhesion-dependent manner [165], but E-cadherin has also been found to activate transiently EGFR when cell–cell contacts are formed [162]. It was demonstrated that E-cadherin alters the ligand (EGF) binding affinity to EGFR and, whenever E-cadherin is deleted (E-cad Δβ-ctn, Ecad AAA(764) and E-cad ΔNT), the interaction of E-cadherin with EGFR is perturbed. In this context E-cadherin impairs EGFR, c-Met and IGF-1R signalling through their cognate receptors [164]. In addition, studies performed using E-cadherin germline missense mutations found in HDGC patients, which are spread throughout the protein, demonstrate that the extracellular domain of E-cadherin is required to interact with EGFR and that this interaction impairs the activation of the receptor by its ligand [159,160]. Binding of E-cadherin to EGFR blocks therefore its downstream signalling, which lead to activation of GTPase RhoA and in consequence increased cell motility [159,160].

Loss of E-cadherin function is also strongly associated with an increased invasive behaviour of the cells. Consistent with this notion, many clinical studies have shown that expression levels of metalloproteases MMP2 and MMP9 correlate with tumour progression [166]. Our group has shown, using *in vitro* models, an involvement of E-cadherin in the regulation of MMP activity. In our case, mutant E-cadherin cells when compared to wild-type cells, endogenously secrete MMP9 (unpublished data). This result is in agreement with previous studies suggesting a relation between E-cadherin and MMP expression [167]. It is interesting to notice that, besides the direct role in cell invasion, MMP activity is known to inactivate E-cadherin by cleavage of its extracellular domain, in a process designated by ectodomain shedding [168–171]. Proteolytic ectodomain fragments of E-cadherin have been proposed to promote cancer cell invasion by interfering with E-cadherin function in cells containing intact E-cadherin/catenin complexes [170,171].

In the process of cellular invasion, and once tumour cells detach from the original tissue, penetrate the basement membrane and invade the interstitial matrix, cell survival depends on signals from the environment, such as those provided by adhesion molecules that mediate contacts between cells or between cells and the surrounding medium [172]. We have demonstrated that loss of functional E-cadherin renders cells more resistant to apoptotic stimuli [173,174]. Cells expressing mutant E-cadherins are not only capable of invading, but are also able to survive in the absence of contact with other cells in a Notch-1 dependent manner [174]. Further, we showed in our *in vitro* model that cells expressing wild-type E-cadherin have a decreased level of the anti-apoptotic Bcl-2, in contrast to mutant forms of the protein, proving the existence of an interplay between E-cadherin and Bcl-2 regulation. Recently, we demonstrated that aberrant expression of Bcl-2 in E-cadherin mutants is dependent on Notch-1 activation [174]. This supports the hypothesis that E-cadherin is also involved in the control of programmed cell death, and thus has a dual role in cancer prevention. Because of the potential therapeutic relevance of this finding, further studies aiming at elucidating its molecular mechanisms are warranted.

3.5. E-cadherin and Helicobacter pylori infection

H. pylori is a bacterial pathogen with a major etiological role in gastric cancer development. Studies have shown that *H. pylori* targets E-cadherin by several mechanisms. Epigenetic silencing of *CDH1* promoter in the context of *H. pylori* infection has been shown using *in vitro* models and in the gastric mucosa of infected patients. *H. pylori* infection of gastric cancer cell lines or treatment with interleukin-1β, a cytokine up-regulated in the context of the infection, induce *CDH1* promoter methylation, leading to decreased E-cadherin expression and concomitant increase in DNA methyltransferase activity [175]. In agreement with the *in vitro* data, *CDH1* promoter methylation has been frequently observed in the gastric mucosa of *H. pylori*-infected patients [176–178], and eradication treatment of the infection reduces *CDH1* promoter methylation levels [177–179]. No *CDH1* mutations have been described to date associated to *H. pylori* infection.

In addition to epigenetic silencing of E-cadherin, *H. pylori* has been associated with other mechanisms of disturbance of E-cadherin functions. *H. pylori* induces extracellular domain cleavage of E-cadherin and both, the host disintegrin metalloproteinase ADAM10 and the *H. pylori* serine protease HtrA, have been implicated in this process [180,181]. These findings are in keeping with a recent report showing that *H. pylori*-positive patients have significantly higher serum levels of soluble E-cadherin than uninfected controls [182].

H. pylori is also involved in the translocation of proteins of the E-cadherin/catenin complex, such as E-cadherin, βctn, and p120ctn, from the cell membrane to intracellular locations in mouse models, primary gastric cells, and different cell lines [183,184]. *H. pylori* induces βctn and p120ctn translocation into the nucleus and transcriptional activation of several LEF/TCF target genes which are important for tumorigenesis [185–188]. These results are, however, not supported by the findings in gastric biopsy specimens from patients with and without *H. pylori* infection, in which no differences were detected in E-cadherin/catenin proteins [189].

Studies from our group have shown that in E-cadherin wild-type cells, *H. pylori* infection promotes the formation of a multiproteic complex containing c-Met and E-cadherin, impairing *H. pylori*-induced c-Met-mediated signalling [190]. In contrast, in E-cadherin defective gastric cancer cells *H. pylori* induces c-Met activation and increases the activity of MMP2 and MMP9, leading to extracellular matrix degradation and subsequent cell invasion [191]. *H. pylori* targeting of the E-cadherin/catenin complex, with loss of cell–cell adhesion and increased cell invasion, may contribute to increase the risk for *H. pylori*-infected individuals to develop gastric cancer.

3.6. E-cadherin in vivo cancer models

The *Drosophila* model has been helpful to uncover the E-cadherin role in invasion. Indeed, when *Drosophila* E-cadherin was overexpressed in $srcib^{-}Ras^{V12}$ cells that had lost E-cadherin expression, it suppressed invasion *in vivo* [192]. Another study from our group has taken advantage of the fly system to effectively direct the expression of different human E-cadherin forms (wild-type or HDGC-associated missense mutants) in the *Drosophila* epithelium, in order to evaluate their biological significance *in vivo*. When HDGC-associated missense

mutants were expressed in the wing epithelium, these molecules mimicked previous results obtained *in vitro* regarding migration and invasion. Moreover, this study sheds new lights on the putative inhibition of Notch signalling by E-cadherin, at least at the level of *cut*, the *Drosophila* orthologue of Cux1 [193]. A similar approach is currently being used in our laboratory to screen for pathways affected by the same human E-cadherin missense mutations in a tissue context [194].

The most compelling evidence for the causal relationship between E-cadherin loss and diffuse gastric cancer initiation, *in vivo*, was reported by Humar et al. in 2009. For the first time, they have successfully induced signet-ring cell carcinomas (SRCCs) in CDH1$^{+/-}$ mice, using a known stomach carcinogen (N-methyl-N-nitrosourea) [195].

Another level of interest was the use of *in vivo* studies unveiling how E-cadherin's loss leads to cancer metastases [21,196,197]. The first evidences showed that nude mice with MDA-MB-231 human breast cancer cells without E-cadherin had a striking enhancement of bone metastases [196]. Also HDGC patients show a high chance of developing ovary metastases. In mice, human cancer cell lines that had lost or downregulated E-cadherin expression were able to metastasise to mice ovaries and the restoring E-cadherin expression led to the complete rescue of the ovarian-metastatic phenotype, but did not interfere with the metastatic capacity to other organs. This data, in mice, showed that E-cadherin regulation is a key step in tissue specific metastases formation, at least in the ovary [198].

4. P-cadherin and cancer

The clinical interest on P-cadherin started some years ago, when Shimoyama et al. examined a large series of lung carcinomas, by immunohistochemistry, and found that P- and E-cadherin were co-expressed in all of them, suggesting that P-cadherin expression was closely related to the differentiation of carcinomas. In contrast to E-cadherin, P-cadherin seemed to be frequently up-regulated in tumours, being reduced when tumour cells were more differentiated [199].

4.1. P-cadherin overexpression

P-cadherin aberrant expression has been already described in many types of tumours, such as oral and oesophageal squamous carcinomas [200], mucoepidermoid carcinomas of the thyroid [201], gastric and pancreatic carcinomas [56,202,203], basal and squamous cell carcinomas of the skin [204], squamous intraepithelial lesions and glandular tumours of the uterine cervix [205,206], endometrial and ovarian carcinomas [207–210], prostate and bladder carcinomas [211,212]. In all these tumours, P-cadherin was preferentially expressed in invasive rather than in *in situ* lesions, showing that aberrant expression of P-cadherin could be a useful marker of invasion capacity of tumour cells.

In this review, special attention is given to the role of P-cadherin in breast cancer, where a high volume of knowledge exists. In breast cancer, P-cadherin is preferentially expressed in breast lesions that show characteristics found in basal-like carcinomas (Fig. 4) [213,214]. We and others have reported that P-cadherin expression in breast carcinomas is inversely related with hormonal receptors content (the majority of the cases are ER and PgR negative) [23,57,215–217], and directly related with the expression of EGFR [218], HER2, p53 expression [23], high proliferation rates (MIB-1) and mitotic index, and decreased cell differentiation, which are biological parameters related with poor prognosis [23,216,219]. In fact, P-cadherin expression is strongly related in breast cancer with a decreased patient survival, especially in a short-term follow-up (first five years), being considered a valuable prognostic factor: the probabilities of disease-free and overall survival were significantly lower for patients with P-cadherin positive tumours [57]. Recently, we also demonstrated that P-cadherin expression has a relevant role in the prognosis of invasive breast cancer that maintains E-cadherin expression, thus can be classified as a biomarker of poor prognosis in E-cadherin positive breast carcinomas. Aberrant P-cadherin expression in E-cadherin positive breast cancer cells is correlated with destabilisation of the normal cadherin/catenin complex, because it is essentially related to p120ctn cytoplasmic expression [220]. Once in the cytoplasm, p120ctn can inhibit RhoA and activate other Rho GTPases, Rac1 and Cdc42, altering the actin cytoskeleton polymerisation and promoting cell migration and motility [56]. This probably explains in part the invasive phenotype of these cells and the poor prognosis. It was also demonstrated that P-cadherin expression is associated to overexpression of matrix metalloproteinases that are relevant in invasion and related with poor prognosis in cancer [91].

As an important pre-screening molecular marker, to suspect from an hereditary form of breast cancer, P-cadherin expression can also be used to distinguish patients who are likely to carry a *BRCA1* germline mutation, thus indicating who should be screened first in families with a high incidence of breast and ovarian cancer [214,221–225]. Gorski et al. demonstrated that BRCA1 is able to regulate the basal phenotype by a transcriptional mechanism, since it represses genes like CK5, CK17, and also *CDH3* (P-cadherin gene). In breast cancer cells with a BRCA1 mutation, this repression does not occur and these genes are codified and expressed [225].

4.2. CDH3 transcriptional deregulation

In 2004, our group explored the link between ER (estrogen receptor) signalling and the regulation of P-cadherin expression in breast cancer cell lines, since we found that breast tumours positive for P-cadherin expression were essentially ER negative. P-cadherin *de novo* expression was shown to result from a lack of ERα signalling. In fact, we verified that P-cadherin expression is able to be induced

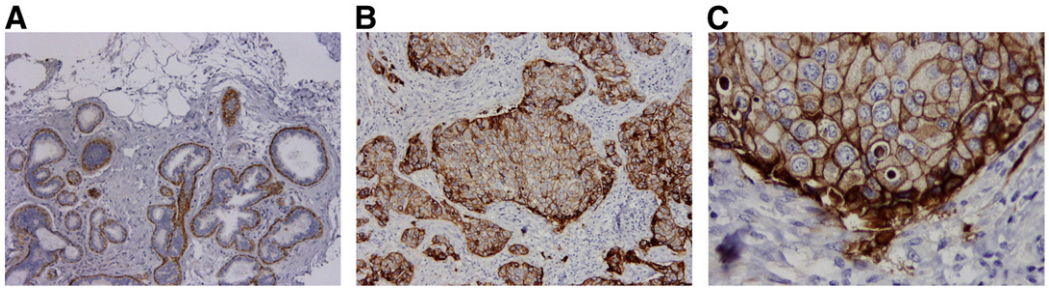


Fig. 4. Immunohistochemistry panel representing the expression pattern of P-cadherin in normal breast tissue and in a breast carcinoma. (A) P-cadherin expression is restricted to the membrane of the myoepithelial cell layer of normal mammary ducts (magnification: 200×); (B) In high grade breast carcinomas, P-cadherin overexpression is homogeneously presented throughout the tumour area (magnification: 200×); (C) In higher magnification, it is clear that P-cadherin is overexpressed at the cell membrane of breast tumour cells (magnification: 400×).

by the pure anti-oestrogen ICI182,780 (ICI) and counteracted by 17β-oestradiol (E2) [57]. Cell lines treated with ICI showed a two to three-fold increase of P-cadherin mRNA and protein levels in a time and dose dependent manner, importantly establishing that the lack of ERα signalling is responsible for the increase of P-cadherin and, therefore, categorising *CDH3* as a putative oestrogen-repressed gene [57]. Very recently, and deeply exploring the ICI-mediated mechanism, we described a cellular adaptation process where ICI is able to induce a chromatin structural remodelling, which leads with the activation of *CDH3* gene and overexpression of P-cadherin protein [226]. Such genomic de-repression effect may contribute to an augmented invasive phenotype of breast cancer cells, which is typically acquired upon endocrine therapy resistance and disease progression. This epigenetic regulatory mechanism contributes to clarify the ability of selective ER modulators and steroidal anti-oestrogens, like fulvestrant (ICI), to induce the inappropriate expression of genes normally repressed by oestrogen/ER signalling pathway.

In 2005, we verified, for the first time, the existence of a significant correlation between hypomethylation of a specific region of *CDH3* gene promoter and P-cadherin overexpression in breast cancer [23]. Moreover, we were able to study normal P-cadherin negative epithelial/luminal breast cells, which were found to be consistently methylated at this *CDH3* promoter region [23]. Interestingly, we showed that, during breast cancer progression, aberrant expression of P-cadherin is due to cytosine demethylation of *CDH3* gene promoter. Using a large series of invasive breast carcinomas, we found that 71% of P-cadherin-negative breast cancer cases were methylated for the *CDH3* gene, whereas 65% of P-cadherin-positive cases were unmethylated [23]. Recently, the epigenetic deregulation of P-cadherin was also demonstrated in other cancer models. Sato et al. identified *CDH3* gene promoter to be aberrantly methylated in 20% of pancreatic cancers, but not in normal pancreatic epithelia [227]. *CDH3* was also shown to be epigenetically deregulated in colorectal cancer; however, in contrast with the pancreatic model, the *CDH3* promoter was found hypomethylated in colonic aberrant crypt foci, in colorectal cancer, and, occasionally, in the normal epithelium adjacent to cancer. This hypomethylation pattern was associated with the induction of P-cadherin expression in the neoplastic colon. Finally, demethylation of the *CDH3* gene was recently detected in 69% of primary gastric carcinomas and was significantly associated with increasing TNM stage, suggesting that the aberrant demethylation of *CDH3* is also a frequent event in gastric carcinomas [228].

Interestingly, it is likely that, in carcinomas with overexpression of P-cadherin, the pivotal molecular mechanism involved in its deregulation is mainly occurring at the promoter region of the gene and not by structural alterations of its coding sequences. Recently, we still found that the CCAAT/enhancer-binding protein β (C/EBPβ) transcription factor was able to activate *CDH3* promoter in breast cancer cells. We showed that this novel activator of *CDH3* promoter activity exerts its action preferably through its truncated LIP isoform, being the abundance of Sp1 sites within the *CDH3* promoter a feature which potentiate the C/EBPβ-LIP activation role on *CDH3* gene [226]. Shimomura and colleagues described p63, a p53-family related transcription factor, as a key regulator of P-cadherin expression [42]. *CDH3*/P-cadherin gene was recently described to be also transcriptionally repressed by functional BRCA1 protein in breast cancer cell lines, at both mRNA and protein levels. Together with BRCA1, the oncogene c-Myc forms a repressor complex on the promoter region of *CDH3* gene [225].

4.3. P-cadherin and signalling pathways

E-cadherin induced signalling pathways have been far studied in cancer; still, little is known about the signalling pathways activated by P-cadherin in cancer. The first limitation is due to P-cadherin dual role, as an invasion promoter or invasion suppressor depending on the cell model under study [56,57,229]. In fact, it has been previously shown that in highly invasive melanoma cell lines (that lack E-cadherin expression), P-cadherin overexpression was able to promote the formation of cell–cell contacts and counteract invasion, like E-cadherin does [229]. It is somehow expected that P-cadherin, acting as an invasion suppressor, share common signalling pathways with the suppressive function of E-cadherin; however, it is not known whether the pathways are triggered in the same way. Interestingly, Sarrió et al. demonstrated that E-cadherin negative breast cancer cells, transfected with E- or P-cadherin, showed an increased number of similarities [230]. According to their data bases, P- or E-cadherin expression in cadherin negative cells had an important impact on the overall genetic program, altering the expression of genes belonging to a wide range of biological functions, including signal transduction and growth factors (VEGFC, FGFR4), cell cycle (CCNA2), cell adhesion and extracellular matrix (CDH4, COL12A1), or cytokines and inflammation (IL24), among others. More recently, the role of P-cadherin was investigated in an oral squamous cancer cell line that was deficient for classical cadherins [231]. After P-cadherin overexpression, GSK-3β was activated leading to phosphorylated Snail and to its cytoplasmic translocation [231]. In the same model system, it was shown that Slit-2, a secreted ECM glycoprotein, facilitates the interaction of P-cadherin with Robo-3, its receptor, and inhibits cell migration [232]. However, in cell systems with endogenous expression of E-cadherin, like breast carcinomas, we noticed that overexpression of P-cadherin induces invasion. The same holds true in situations where there is co-expression of N-cadherin, like HEK293T cells and PDAC pancreatic cancer cells [57]. Using *in vitro* breast cancer cell models, we found that overexpression of P-cadherin promotes single cell motility, directional cell migration, as well as, invasion capacity through Matrigel (unpublished data). This same migratory phenotype was observed in bladder and in pancreatic cancer cell lines [56,233]. One of the molecules that have been several times referred has having a specific role in signalling related to P-cadherin is p120ctn. We demonstrated that the pro-invasive activity of P-cadherin requires the JMD of its cytoplasmic tail. Cells transfected with several mutants of P-cadherin showed that P-cadherin JMD was essential for its invasive behaviour *in vitro* [57]. Moreover, we observed, in tumour samples, that breast carcinomas co-expressing E- and P-cadherin were associated with p120ctn cytoplasmic localisation and poor patient survival, probably because P-cadherin interferes with the normal binding of p120ctn to E-cadherin [220]. From then until now, several other reports have been exploring that pathway. Indeed, Taniuchi et al. showed that the induced cell migration by P-cadherin expression was due to activation of the Rho GTPases, Rac1 and Cdc42, through accumulation of p120ctn in the cytoplasm and cadherin switching in a pancreatic cancer cell model [56]. Very recently, P-cadherin has been also shown to cooperate with insulin-like growth factor-1 receptor to promote metastatic signalling of gonadotropin-releasing hormone in ovarian cancer via p120ctn [234]. These same authors had previously shown that this p120ctn signalling mediated by P-cadherin expression also lead to increased activity levels of Rac1 and Cdc42 [234].

Although binding of proteins at P-cadherin JMD has just been documented for p120ctn [235], other molecules, like Hakai and presenilin-1 (PS-1), have been reported to bind to the JMD of classical cadherins as well, to a sequence adjacent to or overlapping the p120ctn-binding domain, thereby competing with p120ctn for binding [65,236]. Although the significance of these interactions is not well known, we cannot exclude the possibility that disruption of the p120ctn-binding sequence introduces conformational changes and/or uncouples the interaction of these or other proteins, which could be responsible for our observations.

Recently, it has been shown that the regulatory role that P-cadherin has in cell migration is also related with the organisation

of the non-muscle myosin II-B isoform, which is an ATP-dependent molecular motor protein that can interact with and contract filamentous actin (F-actin).These results implicate that there is a coordinated crosstalk between adhesion molecules and cellular migration-related proteins [237].

Additionally, in terms of breast cancer cell invasion, we found that the presence of P-cadherin, in an E-cadherin positive cellular background, is able to provoke the secretion of pro-invasive factors, such as MMP1 and MMP2, which lead to P-cadherin ectodomain cleavage (sP-cad) that also has pro-invasive activity by itself [91]. This study clarified the mechanism associated to invasion and may explain the poor prognosis of breast tumours over-expressing P-cadherin.

For sure, different signalling pathways should be triggered in the different cell models, thus it will be crucial to identify new interaction partners of P-cadherin, as well as to study whether the interaction of known partner molecules differ between cadherins. Finally, it is important to highlight that the effect of cadherins on the overall gene expression program of cancer cells is highly dependent on the cellular type and the biological context. Thus, P-cadherin regulation of specific transcriptional factors may depend on the activation of other signalling pathways, or the presence of additional molecular alterations.

4.4. P-cadherin in vivo cancer models

As previously described, several *in vivo* models have been used to study the role of E-cadherin in cancer; however, for studying the role and function of P-cadherin, only mouse models have been created. Radice and collaborators have done much in trying to investigate the functions of P-cadherin *in vivo*. For that, they mutated the gene encoding this cell adhesion receptor in mice. Interestingly, in contrast to E- and N-cadherin-deficient mice, mice homozygous for the P-cadherin mutation are viable, but develop hyperplasia and dysplasia of the mammary epithelium with age [82]. The same authors developed a transgenic mouse model with forced expression of P-cadherin in the mammary epithelium, under control of the MMTV ?thyc=5?> promoter [238]. These mice did not develop mammary tumours spontaneously, indicating that P-cadherin misexpression by mammary epithelial cells does not induce tumours. However, when mammary tumours were induced in the P-cadherin transgenic mice through a breeding strategy using a transgenic mouse over-expressing ErbB2/HER-2/neu under control of the MMTV promoter, no tumours exhibited P-cadherin expression. This result might have been due to competition between the two MMTV driven transgenes for transcriptional co-factors or to increased adhesion by P-cadherin, acting as a tumour suppressor. Either way, this mouse model did not allow testing the ability of P-cadherin to affect tumour cell behaviour. A future mouse model with an inducible promoter for P-cadherin might resolve the issue of whether or not P-cadherin can enhance tumour cell aggressiveness.

The most recent and different approach was used to study P-cadherin function in colon. A transgenic mice that overexpress P-cadherin specifically in the intestinal and colonic epithelium, under the liver fatty acid binding protein (L-FABP) promoter, was created. Forced ectopic expression of P-cadherin accompanied by Indomethacin-induced inflammation resulted in a three-fold higher crypt fission rate within the small and large intestines in the homozygous mice compared to the wild-type animals ($p<0.02$). The authors concluded that epigenetic demethylation of the P-cadherin promoter in the human intestine permits its ectopic expression very early in the colorectal adenoma-carcinoma sequence and persists during invasive cancer. Induced P-cadherin expression, especially in mucosal damage, leads to an increased rate of crypt fission, a common feature of clonal expansion in gastrointestinal dysplasia [228].

5. E- and P-cadherin related molecules for therapeutic use in cancer

Addressing novel targets and the application of individualised management should be the way to improve the long term survival and quality of life for cancer patients, namely those associated to complete E-cadherin loss of function. An apparent attractive approach for treatment of the disease would be the reconstitution of E-cadherin expression. This would be possible in cases with E-cadherin promoter methylation using either well described pleotropic epigenetic drugs, which constitute an attractive possibility for disease control, because they inhibit histone deacetylases (HDAC) and DNA methyltransferases and restore gene expression, or the recently described strategy for specific gene promoter demethylation [239]. The later is based on direct targeting of thymine-DNA-glycosylase (TDG) to specific sequences in the DNA and result in local DNA demethylation at potential regulatory sequences and lead to enhanced gene induction [239]. The identification of E-cadherin molecular targets is therefore mandatory. Despite that, the signalling pathways that are aberrantly activated in consequence of E-cadherin loss, observed in the progression of epithelial cancers, remain elusive in a significant percentage of cases. In this report, we describe that HER receptors (HER2/neu and EGFR) and Notch downstream targets are aberrantly activated in primary gastric cancer with loss of E-cadherin. Aberrantly activated signalling molecules interacting with E-cadherin represent the ideal targets for the development of therapeutic agents. Since EGFR and Notch inhibitors, as well as for their downstream molecules, are already commercially available and some of them in clinical use for the treatment of diverse tumour models, we strongly believe that these targets and associated pathways will create the basis for the development of new therapeutic control in E-cadherin-mediated cancer.

In contrast to the suppressor role of E-cadherin in its wild-type form, we describe P-cadherin as a pro-invasive molecule in the context of tumours with wild-type E-cadherin expression at the membrane of the same cell, as verified in breast carcinomas with poor prognosis. In this particular case, we are strongly convinced that P-cadherin itself or specific targets of P-cadherin are likely to contribute to improve the long term survival and quality of life of cancer patients. Recently, a novel and highly selective human monoclonal antibody against P-cadherin was produced—PF-03732010, which has showed anti-tumour and anti-metastatic activity in a diverse panel of P-cadherin-over-expressing tumour models, without introducing any adverse secondary effects in mice [240]. This IgG failed to bind to the most closely target-related family members, including E-cadherin, N-cadherin, and VE-cadherin. PF-03732010 reduced lymph node metastases and lowered the levels of circulating tumour cells (CTC) in whole blood of P-cadherin-positive tumour bearing mice. The anti-metastatic property of the antibody was remarkable, since it significantly inhibited tumour cell infiltration into the lungs. PF-03732010 still suppressed βctn, cyclin D1, Vimentin, Bcl-2 and survivin expression, decreased the Ki67 levels, and increased caspase-3 expression [240].

Taking all data collected till now, we propose that signalling pathways aberrantly activated in cancer by overexpression of P-cadherin in an E-cadherin cell background or by loss of E-cadherin, both leading to E-cadherin dysfunction, represent the ideal targets for the development of novel therapeutic biological agents.

Acknowledgements

We thank the Portuguese Foundation for Science and Technology (FCT) (Projects: PTDC/SAU-ONC/110294/2009; PTDC/SAU-GMG/120049/2010; PTDC/SAU-GMG/72168/2006, PTDC/SAU-GMG/110785/2009; PTDC/CVT/111358/2009; and PTDC/BIA-MIC/116890/2010). Salary support to JP, CO and MJO from POPH-QREN/Type 4.2, the European Social Fund and the Portuguese Ministry of Science and Technology (MCTES). IPATIMUP is an Associate Laboratory of the Portuguese

Ministry of Science, Technology and Higher Education and is partially supported by the FCT.

References

[1] U. Cavallaro, G. Christofori, Cell adhesion and signalling by cadherins and Ig-CAMs in cancer, Nat. Rev. Cancer 4 (2004) 118–132.
[2] K. Gerrow, A. El-Husseini, Cell adhesion molecules at the synapse, Front. Biosci. 11 (2006) 2400–2419.
[3] F. Nollet, P. Kools, F. van Roy, Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members, J. Mol. Biol. 299 (2000) 551–572.
[4] M. Takeichi, Cadherins in cancer: implications for invasion and metastasis, Curr. Opin. Cell Biol. 5 (1993) 806–811.
[5] M. Pignatelli, W.F. Bodmer, Genetics and biochemistry of collagen binding-triggered glandular differentiation in a human colon carcinoma cell line, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 5561–5565.
[6] M. Takeichi, Morphogenetic roles of classic cadherins, Curr. Opin. Cell Biol. 7 (1995) 619–627.
[7] L. Shapiro, A.M. Fannon, P.D. Kwong, A. Thompson, M.S. Lehmann, G. Grubel, J.F. Legrand, J. Als-Nielsen, D.R. Colman, W.A. Hendrickson, Structural basis of cell–cell adhesion by cadherins, Nature 374 (1995) 327–337.
[8] M. Ozawa, M. Ringwald, R. Kemler, Uvomorulin–catenin complex formation is regulated by a specific domain in the cytoplasmic region of the cell adhesion molecule, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4246–4250.
[9] O.W. Blaschuk, R. Sullivan, S. David, Y. Pouliot, Identification of a cadherin cell adhesion recognition sequence, Dev. Biol. 139 (1990) 227–229.
[10] M. Ozawa, H. Baribault, R. Kemler, The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species, EMBO J. 8 (1989) 1711–1717.
[11] B.M. Gumbiner, P.D. McCrea, Catenins as mediators of the cytoplasmic functions of cadherins, J. Cell Sci. Suppl. 17 (1993) 155–158.
[12] A.S. Yap, C.M. Niessen, B.M. Gumbiner, The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn, J. Cell Biol. 141 (1998) 779–789.
[13] E. Van Aken, O. De Wever, A.S. Correia da Rocha, M. Mareel, Defective E-cadherin/catenin complexes in human cancer, Virchows Arch. 439 (2001) 725–751.
[14] M. Mareel, M.J. Oliveira, I. Madani, Cancer invasion and metastasis: interacting ecosystems, Virchows Arch. 454 (2009) 599–622.
[15] N. Makrilia, A. Kollias, L. Manolopoulos, K. Syrigos, Cell adhesion molecules: role and clinical significance in cancer, Cancer Invest. 27 (2009) 1023–1037.
[16] M.J. Bussemakers, A. van Bokhoven, M. Voller, F.P. Smit, J.A. Schalken, The genes for the calcium-dependent cell adhesion molecules P- and E-cadherin are tandemly arranged in the human genome, Biochem. Biophys. Res. Commun. 203 (1994) 1291–1294.
[17] G. Berx, K. Staes, J. van Hengel, F. Molemans, M.J. Bussemakers, A. van Bokhoven, F. van Roy, Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1), Genomics 26 (1995) 281–289.
[18] D.F. Jarrard, R. Paul, A. van Bokhoven, S.H. Nguyen, G.S. Bova, M.J. Wheelock, K.R. Johnson, J. Schalken, M. Bussemakers, W.B. Isaacs, P-cadherin is a basal cell-specific epithelial marker that is not expressed in prostate cancer, Clin. Cancer Res. 3 (1997) 2121–2128.
[19] T.J. Hubbard, B.L. Aken, S. Ayling, B. Ballester, K. Beal, E. Bragin, S. Brent, Y. Chen, P. Clapham, L. Clarke, G. Coates, S. Fairley, S. Fitzgerald, J. Fernandez-Banet, L. Gordon, S. Graf, S. Haider, M. Hammond, R. Holland, K. Howe, A. Jenkinson, N. Johnson, A. Kahari, D. Keefe, S. Keenan, R. Kinsella, F. Kokocinski, E. Kulesha, D. Lawson, I. Longden, K. Megy, P. Meidl, B. Overduin, A. Parker, B. Pritchard, D. Rios, M. Schuster, G. Slater, D. Smedley, W. Spooner, G. Spudich, S. Trevanion, A. Vilella, J. Vogel, S. White, S. Wilder, A. Zadissa, E. Birney, F. Cunningham, V. Curwen, R. Durbin, X.M. Fernandez-Suarez, J. Herrero, A. Kasprzyk, G. Proctor, J. Smith, S. Searle, P. Flicek, Ensembl 2009, Nucleic Acids Res. 37 (2009) D690–D697.
[20] M.L. Faraldo, A. Cano, The 5′ flanking sequences of the mouse P-cadherin gene. Homologies to 5′ sequences of the E-cadherin gene and identification of a first 215 base-pair intron, J. Mol. Biol. 231 (1993) 935–941.
[21] F. van Roy, G. Berx, The cell–cell adhesion molecule E-cadherin, Cell. Mol. Life Sci. 65 (2008) 3756–3788.
[22] M.P. Stemmler, Cadherins in development and cancer, Mol. Biosyst. 4 (2008) 835–850.
[23] J. Paredes, A. Albergaria, J.T. Oliveira, C. Jeronimo, F. Milanezi, F.C. Schmitt, P-cadherin overexpression is an indicator of clinical outcome in invasive breast carcinomas and is associated with CDH3 promoter hypomethylation, Clin. Cancer Res. 11 (2005) 5869–5877.
[24] K.R. Sakharkar, V.T. Chow, PPD—proteome profile database, In Silico Biol. 4 (2004) 219–223.
[25] D.J. Thomas, K.R. Rosenbloom, H. Clawson, A.S. Hinrichs, H. Trumbower, B.J. Raney, D. Karolchik, G.P. Barber, R.A. Harte, J. Hillman-Jackson, R.M. Kuhn, B.L. Rhead, K.E. Smith, A. Thakkapallayil, A.S. Zweig, D. Haussler, W.J. Kent, The ENCODE Project at UC Santa Cruz, Nucleic Acids Res. 35 (2007) D663–D667.
[26] P. Oliveira, R. Sanges, D. Huntsman, E. Stupka, C. Oliveira, Characterization of the intronic portion of cadherin superfamily members, common cancer orchestrators, Eur. J. Hum. Genet. (2012), http://dx.doi.org/10.1038/ejhg.2012.11.
[27] S. Hosono, I. Gross, M.A. English, K.M. Hajra, E.R. Fearon, J.D. Licht, E-cadherin is a WT1 target gene, J. Biol. Chem. 275 (2000) 10943–10953.
[28] Y.N. Liu, W.W. Lee, C.Y. Wang, T.H. Chao, Y. Chen, J.H. Chen, Regulatory mechanisms controlling human E-cadherin gene expression, Oncogene 24 (2005) 8277–8290.
[29] J. Behrens, O. Lowrick, L. Klein-Hitpass, W. Birchmeier, The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11495–11499.
[30] A. Cano, M.A. Perez-Moreno, I. Rodrigo, A. Locascio, M.J. Blanco, M.G. del Barrio, F. Portillo, M.A. Nieto, The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression, Nat. Cell Biol. 2 (2000) 76–83.
[31] K.M. Hajra, D.Y. Chen, E.R. Fearon, The SLUG zinc-finger protein represses E-cadherin in breast cancer, Cancer Res. 62 (2002) 1613–1618.
[32] M.A. Perez-Moreno, A. Locascio, I. Rodrigo, G. Dhondt, F. Portillo, M.A. Nieto, A. Cano, A new role for E12/E47 in the repression of E-cadherin expression and epithelial–mesenchymal transitions, J. Biol. Chem. 276 (2001) 27424–27431.
[33] J. Comijn, G. Berx, P. Vermassen, K. Verschueren, L. van Grunsven, E. Bruyneel, M. Mareel, D. Huylebroeck, F. van Roy, The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion, Mol. Cell 7 (2001) 1267–1278.
[34] A. Eger, K. Aigner, S. Sonderegger, B. Dampier, S. Oehler, M. Schreiber, G. Berx, A. Cano, H. Beug, R. Foisner, DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells, Oncogene 24 (2005) 2375–2385.
[35] H. Peinado, D. Olmeda, A. Cano, Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat. Rev. Cancer 7 (2007) 415–428.
[36] Y. Shi, J. Sawada, G. Sui, B. Affar el, J.R. Whetstine, F. Lan, H. Ogawa, M.P. Luke, Y. Nakatani, Coordinated histone modifications mediated by a CtBP co-repressor complex, Nature 422 (2003) 735–738.
[37] C. Jamora, R. DasGupta, P. Kocieniewski, E. Fuchs, Links between signal transduction, transcription and adhesion in epithelial bud development, Nature 422 (2003) 317–322.
[38] C. Vandewalle, J. Comijn, B. De Craene, P. Vermassen, E. Bruyneel, H. Andersen, E. Tulchinsky, F. Van Roy, G. Berx, SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell–cell junctions, Nucleic Acids Res. 33 (2005) 6566–6578.
[39] K. Aigner, B. Dampier, L. Descovich, M. Mikula, A. Sultan, M. Schreiber, W. Mikulits, T. Brabletz, D. Strand, P. Obrist, W. Sommergruber, N. Schweifer, A. Wernitznig, H. Beug, R. Foisner, A. Eger, The transcription factor ZEB1 (deltaEF1) promotes tumour cell dedifferentiation by repressing master regulators of epithelial polarity, Oncogene 26 (2007) 6979–6988.
[40] S. Spaderna, O. Schmalhofer, M. Wahlbuhl, A. Dimmler, K. Bauer, A. Sultan, F. Hlubek, A. Jung, D. Strand, A. Eger, T. Kirchner, J. Behrens, T. Brabletz, The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer, Cancer Res. 68 (2008) 537–544.
[41] J.R. Graff, J.G. Herman, R.G. Lapidus, H. Chopra, R. Xu, D.F. Jarrard, W.B. Isaacs, P.M. Pitha, N.E. Davidson, S.B. Baylin, E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas, Cancer Res. 55 (1995) 5195–5199.
[42] Y. Shimomura, M. Wajid, L. Shapiro, A.M. Christiano, P-cadherin is a p63 target gene with a crucial role in the developing human limb bud and hair follicle, Development 135 (2008) 743–753.
[43] D.K. Carroll, J.S. Carroll, C.O. Leong, F. Cheng, M. Brown, A.A. Mills, J.S. Brugge, L.W. Ellisen, p63 regulates an adhesion programme and cell survival in epithelial cells, Nat. Cell Biol. 8 (2006) 551–561.
[44] M.M. Faraldo, J. Teuliere, M.A. Deugnier, W. Birchmeier, J. Huelsken, J.P. Thiery, A. Cano, M.A. Glukhova, Beta-catenin regulates P-cadherin expression in mammary basal epithelial cells, FEBS Lett. 581 (2007) 831–836.
[45] T.L. Le, A.S. Yap, J.L. Stow, Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics, J. Cell Biol. 146 (1999) 219–232.
[46] D.M. Bryant, J.L. Stow, The ins and outs of E-cadherin trafficking, Trends Cell Biol. 14 (2004) 427–434.
[47] L. Hinck, I.S. Nathke, J. Papkoff, W.J. Nelson, Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly, J. Cell Biol. 125 (1994) 1327–1340.
[48] Y.T. Chen, D.B. Stewart, W.J. Nelson, Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and basal–lateral membrane targeting of E-cadherin in polarized MDCK cells, J. Cell Biol. 144 (1999) 687–699.
[49] J.G. Lock, J.L. Stow, Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin, Mol. Biol. Cell 16 (2005) 1744–1755.
[50] D.M. Bryant, M.C. Kerr, L.A. Hammond, S.R. Joseph, K.E. Mostov, R.D. Teasdale, J.L. Stow, EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin, J. Cell Sci. 120 (2007) 1818–1828.
[51] K. Ling, S.F. Bairstow, C. Carbonara, D.A. Turbin, D.G. Huntsman, R.A. Anderson, Type I gamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin, J. Cell Biol. 176 (2007) 343–353.
[52] Y. Mosesson, G.B. Mills, Y. Yarden, Derailed endocytosis: an emerging feature of cancer, Nat. Rev. Cancer 8 (2008) 835–850.
[53] R.C. Ireton, M.A. Davis, J. van Hengel, D.J. Mariner, K. Barnes, M.A. Thoreson, P.Z. Anastasiadis, L. Matrisian, L.M. Bundy, L. Sealy, B. Gilbert, F. van Roy, A.B. Reynolds, A novel role for p120 catenin in E-cadherin function, J. Cell Biol. 159 (2002) 465–476.
[54] M.A. Davis, R.C. Ireton, A.B. Reynolds, A core function for p120-catenin in cadherin turnover, J. Cell Biol. 163 (2003) 525–534.

[55] K. Xiao, J. Garner, K.M. Buckley, P.A. Vincent, C.M. Chiasson, E. Dejana, V. Faundez, A.P. Kowalczyk, p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin, Mol. Biol. Cell 16 (2005) 5141–5151.
[56] K. Taniuchi, H. Nakagawa, M. Hosokawa, T. Nakamura, H. Eguchi, H. Ohigashi, O. Ishikawa, T. Katagiri, Y. Nakamura, Overexpressed P-cadherin/CDH3 promotes motility of pancreatic cancer cells by interacting with p120ctn and activating rho-family GTPases, Cancer Res. 65 (2005) 3092–3099.
[57] J. Paredes, C. Stove, V. Stove, F. Milanezi, V. Van Marck, L. Derycke, M. Mareel, M. Bracke, F. Schmitt, P-cadherin is up-regulated by the antiestrogen ICI 182,780 and promotes invasion of human breast cancer cells, Cancer Res. 64 (2004) 8309–8317.
[58] F. Palacios, L. Price, J. Schweitzer, J.G. Collard, C. D'Souza-Schorey, An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration, EMBO J. 20 (2001) 4973–4986.
[59] J. Figueiredo, J. Simoes-Correia, O. Soderberg, G. Suriano, R. Seruca, ADP-ribosylation factor 6 mediates E-cadherin recovery by chemical chaperones, PLoS One 6 (2011) e23188.
[60] F. Palacios, J.K. Schweitzer, R.L. Boshans, C. D'Souza-Schorey, ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly, Nat. Cell Biol. 4 (2002) 929–936.
[61] Y. Otsuki, M. Tanaka, S. Yoshii, N. Kawazoe, K. Nakaya, H. Sugimura, Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 4385–4390.
[62] M. Morishige, S. Hashimoto, E. Ogawa, Y. Toda, H. Kotani, M. Hirose, S. Wei, A. Hashimoto, A. Yamada, H. Yano, Y. Mazaki, H. Kodama, Y. Nio, T. Manabe, H. Wada, H. Kobayashi, H. Sabe, GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion, Nat. Cell Biol. 10 (2008) 85–92.
[63] J.M. Daniel, A.B. Reynolds, Tyrosine phosphorylation and cadherin/catenin function, Bioessays 19 (1997) 883–891.
[64] F. Palacios, J.S. Tushir, Y. Fujita, C. D'Souza-Schorey, Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell–cell adhesion during epithelial to mesenchymal transitions, Mol. Cell. Biol. 25 (2005) 389–402.
[65] Y. Fujita, G. Krause, M. Scheffner, D. Zechner, H.E. Leddy, J. Behrens, T. Sommer, W. Birchmeier, Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex, Nat. Cell Biol. 4 (2002) 222–231.
[66] S.S. Pinho, R. Seruca, F. Gartner, Y. Yamaguchi, J. Gu, N. Taniguchi, C.A. Reis, Modulation of E-cadherin function and dysfunction by N-glycosylation, Cell. Mol. Life Sci. 68 (2011) 1011–1020.
[67] W. Zhu, B. Leber, D.W. Andrews, Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis, EMBO J. 20 (2001) 5999–6007.
[68] F. Zhou, J. Su, L. Fu, Y. Yang, L. Zhang, L. Wang, H. Zhao, D. Zhang, Z. Li, X. Zha, Unglycosylation at Asn-633 made extracellular domain of E-cadherin folded incorrectly and arrested in endoplasmic reticulum, then sequentially degraded by ERAD, Glycoconj. J. 25 (2008) 727–740.
[69] A. Liwosz, T. Lei, M.A. Kukuruzinska, N-glycosylation affects the molecular organization and stability of E-cadherin junctions, J. Biol. Chem. 281 (2006) 23138–23149.
[70] S.S. Pinho, C.A. Reis, J. Paredes, A.M. Magalhaes, A.C. Ferreira, J. Figueiredo, W. Xiaogang, F. Carneiro, F. Gartner, R. Seruca, The role of N-acetylglucosaminyltransferase III and V in the post-transcriptional modifications of E-cadherin, Hum. Mol. Genet. 18 (2009) 2599–2608.
[71] S.S. Pinho, P. Oliveira, J. Cabral, S. Carvalho, D. Huntsman, F. Gartner, R. Seruca, C.A. Reis, C. Oliveira, Loss and recovery of Mgat3 and GnT-III mediated E-cadherin N-glycosylation is a mechanism involved in epithelial–mesenchymal–epithelial transitions, PLoS One 7 (2012) e33191.
[72] D. Osumi, M. Takahashi, E. Miyoshi, S. Yokoe, S.H. Lee, K. Noda, S. Nakamori, J. Gu, Y. Ikeda, Y. Kuroki, K. Sengoku, M. Ishikawa, N. Taniguchi, Core fucosylation of E-cadherin enhances cell–cell adhesion in human colon carcinoma WiDr cells, Cancer Sci. 100 (2009) 888–895.
[73] M. Takeichi, Functional correlation between cell adhesive properties and some cell surface proteins, J. Cell Biol. 75 (1977) 464–474.
[74] F. Hyafil, D. Morello, C. Babinet, F. Jacob, A cell surface glycoprotein involved in the compaction of embryonal carcinoma cells and cleavage stage embryos, Cell 21 (1980) 927–934.
[75] S. Ogou, T.S. Okada, M. Takeichi, Cleavage stage mouse embryos share a common cell adhesion system with teratocarcinoma cells, Dev. Biol. 92 (1982) 521–528.
[76] M. Takeichi, The cadherins: cell–cell adhesion molecules controlling animal morphogenesis, Development 102 (1988) 639–655.
[77] T.P. Fleming, Q. Javed, M. Hay, Epithelial differentiation and intercellular junction formation in the mouse early embryo, Dev. Suppl. (1992) 105–112.
[78] J.D. Aplin, C.J. Jones, L.K. Harris, Adhesion molecules in human trophoblast—a review. I. Villous trophoblast, Placenta 30 (2009) 293–298.
[79] A. Nose, M. Takeichi, A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos, J. Cell Biol. 103 (1986) 2649–2658.
[80] L. Larue, M. Ohsugi, J. Hirchenhain, R. Kemler, E-cadherin null mutant embryos fail to form a trophectoderm epithelium, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 8263–8267.
[81] D. Riethmacher, V. Brinkmann, C. Birchmeier, A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 855–859.
[82] G.L. Radice, M.C. Ferreira-Cornwell, S.D. Robinson, H. Rayburn, L.A. Chodosh, M. Takeichi, R.O. Hynes, Precocious mammary gland development in P-cadherin-deficient mice, J. Cell Biol. 139 (1997) 1025–1032.
[83] T. Frebourg, C. Oliveira, P. Hochain, R. Karam, S. Manouvrier, C. Graziadio, M. Vekemans, A. Hartmann, S. Baert-Desurmont, C. Alexandre, S. Lejeune Dumoulin, C. Marroni, C. Martin, S. Castedo, M. Lovett, J. Winston, J.C. Machado, T. Attie, E.W. Jabs, J. Cai, P. Pellerin, J.P. Triboulet, M. Scotte, F. Le Pessot, A. Hedouin, F. Carneiro, M. Blayau, R. Seruca, Cleft lip/palate and CDH1/E-cadherin mutations in families with hereditary diffuse gastric cancer, J. Med. Genet. 43 (2006) 138–142.
[84] K.W. Kjaer, L. Hansen, G.C. Schwabe, A.P. Marques-de-Faria, H. Eiberg, S. Mundlos, N. Tommerup, T. Rosenberg, Distinct CDH3 mutations cause ectodermal dysplasia, ectrodactyly, macular dystrophy (EEM syndrome), J. Med. Genet. 42 (2005) 292–298.
[85] E. Sprecher, R. Bergman, G. Richard, R. Lurie, S. Shalev, D. Petronius, A. Shalata, Y. Anbinder, R. Leibu, I. Perlman, N. Cohen, R. Szargel, Hypotrichosis with juvenile macular dystrophy is caused by a mutation in CDH3, encoding P-cadherin, Nat. Genet. 29 (2001) 134–136.
[86] W.J. Nelson, Epithelial cell polarity from the outside looking in, News Physiol. Sci. 18 (2003) 143–146.
[87] Y. Hirai, A. Nose, S. Kobayashi, M. Takeichi, Expression and role of E- and P-cadherin adhesion molecules in embryonic histogenesis. II. Skin morphogenesis, Development 105 (1989) 271–277.
[88] G. Berx, F. Van Roy, The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression, Breast Cancer Res. 3 (2001) 289–293.
[89] Y. Shimoyama, S. Hirohashi, Expression of E- and P-cadherin in gastric carcinomas, Cancer Res. 51 (1991) 2185–2192.
[90] M.D. Hines, H.C. Jin, M.J. Wheelock, P.J. Jensen, Inhibition of cadherin function differentially affects markers of terminal differentiation in cultured human keratinocytes, J. Cell Sci. 112 (Pt 24) (1999) 4569–4579.
[91] A.S. Ribeiro, A. Albergaria, B. Sousa, A.L. Correia, M. Bracke, R. Seruca, F.C. Schmitt, J. Paredes, Extracellular cleavage and shedding of P-cadherin: a mechanism underlying the invasive behaviour of breast cancer cells, Oncogene 29 (2010) 392–402.
[92] R. Moll, M. Mitze, U.H. Frixen, W. Birchmeier, Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas, Am. J. Pathol. 143 (1993) 1731–1742.
[93] C. Gamallo, J. Palacios, A. Suarez, A. Pizarro, P. Navarro, M. Quintanilla, A. Cano, Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma, Am. J. Pathol. 142 (1993) 987–993.
[94] J.S. Reis-Filho, J. Cancela Paredes, F. Milanezi, F.C. Schmitt, Clinicopathologic implications of E-cadherin reactivity in patients with lobular carcinoma in situ of the breast, Cancer 94 (2002) 2114–2115.
author reply 2115–2116.
[95] B. Weigelt, F.C. Geyer, R. Natrajan, M.A. Lopez-Garcia, A.S. Ahmad, K. Savage, B. Kreike, J.S. Reis-Filho, The molecular underpinning of lobular histological growth pattern: a genome-wide transcriptomic analysis of invasive lobular carcinomas and grade- and molecular subtype-matched invasive ductal carcinomas of no special type, J. Pathol. 220 (2010) 45–57.
[96] P.T. Simpson, J.S. Reis-Filho, M.B. Lambros, C. Jones, D. Steele, A. Mackay, M. Iravani, K. Fenwick, T. Dexter, A. Jones, L. Reid, L. Da Silva, S.J. Shin, D. Hardisson, A. Ashworth, F.C. Schmitt, J. Palacios, S.R. Lakhani, Molecular profiling pleomorphic lobular carcinomas of the breast: evidence for a common molecular genetic pathway with classic lobular carcinomas, J. Pathol. 215 (2008) 231–244.
[97] B. Mahler-Araujo, K. Savage, S. Parry, J.S. Reis-Filho, Reduction of E-cadherin expression is associated with non-lobular breast carcinomas of basal-like and triple negative phenotype, J. Clin. Pathol. 61 (2008) 615–620.
[98] N. Lopes, J. Carvalho, C. Duraes, B. Sousa, M. Gomes, J.L. Costa, C. Oliveira, J. Paredes, F. Schmitt, 1Alpha,25-dihydroxyvitamin D3 induces de novo E-cadherin expression in triple-negative breast cancer cells by CDH1-promoter demethylation, Anticancer. Res. 32 (2012) 249–257.
[99] G. Berx, A.M. Cleton-Jansen, K. Strumane, W.J. de Leeuw, F. Nollet, F. van Roy, C. Cornelisse, E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain, Oncogene 13 (1996) 1919–1925.
[100] G. Keller, H. Vogelsang, I. Becker, J. Hutter, K. Ott, S. Candidus, T. Grundei, K.F. Becker, J. Mueller, J.R. Siewert, H. Hofler, Diffuse type gastric and lobular breast carcinoma in a familial gastric cancer patient with an E-cadherin germline mutation, Am. J. Pathol. 155 (1999) 337–342.
[101] K.A. Schrader, S. Masciari, N. Boyd, S. Wiyrick, P. Kaurah, J. Senz, W. Burke, H.T. Lynch, J.E. Garber, D.G. Huntsman, Hereditary diffuse gastric cancer: association with lobular breast cancer, Fam. Cancer 7 (2008) 73–82.
[102] K.F. Becker, M.J. Atkinson, U. Reich, I. Becker, H. Nekarda, J.R. Siewert, H. Hofler, E-cadherin gene mutations provide clues to diffuse type gastric carcinomas, Cancer Res. 54 (1994) 3845–3852.
[103] K.F. Becker, E. Kremmer, M. Eulitz, I. Becker, G. Handschuh, C. Schuhmacher, W. Muller, H.E. Gabbert, A. Ochiai, S. Hirohashi, H. Hofler, Analysis of E-cadherin in diffuse-type gastric cancer using a mutation-specific monoclonal antibody, Am. J. Pathol. 155 (1999) 1803–1809.
[104] J.C. Machado, F. Carneiro, S. Beck, S. Rossi, J. Lopes, A. Taveira-Gomes, M. Cardoso-Oliveira, E-cadherin expression is correlated with the isolated cell/diffuse histotype and with features of biological aggressiveness of gastric carcinoma, Int. J. Surg. Pathol. 6 (1998) 135–144.
[105] F. Carneiro, J.C. Machado, R. Seruca, M. Sobrinho-Simoes, E-cadherin changes in gastric carcimona, Histopathology 35 (1999) 477–478.
[106] J. Carvalho, N.C. van Grieken, P.M. Pereira, S. Sousa, M. Tijssen, T.E. Buffart, B. Diosdado, H. Grabsch, M.A. Santos, G. Meijer, R. Seruca, B. Carvalho, C. Oliveira,

Lack of microRNA-101 causes E-cadherin functional deregulation through EZH2 upregulation in intestinal gastric cancer, J. Pathol. (2012), http://dx.doi.org/10.1002/path.4032.
[107] J.C. Machado, C. Oliveira, R. Carvalho, P. Soares, G. Berx, C. Caldas, R. Seruca, F. Carneiro, M. Sobrinho-Simoes, E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma, Oncogene 20 (2001) 1525–1528.
[108] P. Guilford, J. Hopkins, J. Harraway, M. McLeod, N. McLeod, P. Harawira, H. Taite, R. Scoular, A. Miller, A.E. Reeve, E-cadherin germline mutations in familial gastric cancer, Nature 392 (1998) 402–405.
[109] C. Oliveira, J. Senz, P. Kaurah, H. Pinheiro, R. Sanges, A. Haegert, G. Corso, J. Schouten, R. Fitzgerald, H. Vogelsang, G. Keller, S. Dwerryhouse, D. Grimmer, S.F. Chin, H.K. Yang, C.E. Jackson, R. Seruca, F. Roviello, E. Stupka, C. Caldas, D. Huntsman, Germline CDH1 deletions in hereditary diffuse gastric cancer families, Hum. Mol. Genet. 18 (2009) 1545–1555.
[110] H. Pinheiro, R. Bordeira-Carrico, S. Seixas, J. Carvalho, J. Senz, P. Oliveira, P. Inacio, L. Gusmao, J. Rocha, D. Huntsman, R. Seruca, C. Oliveira, Allele-specific CDH1 downregulation and hereditary diffuse gastric cancer, Hum. Mol. Genet. 19 (2010) 943–952.
[111] P. Guilford, B. Humar, V. Blair, Hereditary diffuse gastric cancer: translation of CDH1 germline mutations into clinical practice, Gastric Cancer 13 (2010) 1–10.
[112] G. Suriano, S. Seixas, J. Rocha, R. Seruca, A model to infer the pathogenic significance of CDH1 germline missense variants, J. Mol. Med. 84 (2006) 1023–1031.
[113] G. Suriano, C. Oliveira, P. Ferreira, J.C. Machado, M.C. Bordin, O. De Wever, E.A. Bruyneel, N. Moguilevsky, N. Grehan, T.R. Porter, F.M. Richards, R.H. Hruban, F. Roviello, D. Huntsman, M. Mareel, F. Carneiro, C. Caldas, R. Seruca, Identification of CDH1 germline missense mutations associated with functional inactivation of the E-cadherin protein in young gastric cancer probands, Hum. Mol. Genet. 12 (2003) 575–582.
[114] G. Suriano, M.J. Oliveira, D. Huntsman, A.R. Mateus, P. Ferreira, F. Casares, C. Oliveira, F. Carneiro, J.C. Machado, M. Mareel, R. Seruca, E-cadherin germline missense mutations and cell phenotype: evidence for the independence of cell invasion on the motile capabilities of the cells, Hum. Mol. Genet. 12 (2003) 3007–3016.
[115] A.R. Brooks-Wilson, P. Kaurah, G. Suriano, S. Leach, J. Senz, N. Grehan, Y.S. Butterfield, J. Jeyes, J. Schinas, J. Bacani, M. Kelsey, P. Ferreira, B. MacGillivray, P. MacLeod, M. Micek, J. Ford, W. Foulkes, K. Australie, C. Greenberg, M. LaPointe, C. Gilpin, S. Nikkel, D. Gilchrist, R. Hughes, C.E. Jackson, K.G. Monaghan, M.J. Oliveira, R. Seruca, S. Gallinger, C. Caldas, D. Huntsman, Germline E-cadherin mutations in hereditary diffuse gastric cancer: assessment of 42 new families and review of genetic screening criteria, J. Med. Genet. 41 (2004) 508–517.
[116] P. Kaurah, A. MacMillan, N. Boyd, J. Senz, A. De Luca, N. Chun, G. Suriano, S. Zaor, L. Van Manen, C. Gilpin, S. Nikkel, M. Connolly-Wilson, S. Weissman, W.S. Rubinstein, C. Sebold, R. Greenstein, J. Stroop, D. Yim, B. Panzini, W. McKinnon, M. Greenblatt, D. Wirtzfeld, D. Fontaine, D. Coit, S. Yoon, D. Chung, G. Lauwers, A. Pizzuti, C. Vaccaro, M.A. Redal, C. Oliveira, M. Tischkowitz, S. Olschwang, S. Gallinger, H. Lynch, J. Green, J. Ford, P. Pharoah, B. Fernandez, D. Huntsman, Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer, JAMA 297 (2007) 2360–2372.
[117] G. Corso, F. Roviello, J. Paredes, C. Pedrazzani, M. Novais, J. Correia, D. Marrelli, L. Cirnes, R. Seruca, C. Oliveira, G. Suriano, Characterization of the P373L E-cadherin germline missense mutation and implication for clinical management, Eur. J. Surg. Oncol. 33 (2007) 1061–1067.
[118] H. More, B. Humar, W. Weber, R. Ward, A. Christian, C. Lintott, F. Graziano, A.M. Ruzzo, E. Acosta, B. Boman, M. Harlan, P. Ferreira, R. Seruca, G. Suriano, P. Guilford, Identification of seven novel germline mutations in the human E-cadherin (CDH1) gene, Hum. Mutat. 28 (2007) 203.
[119] C. Oliveira, J. de Bruin, S. Nabais, M. Ligtenberg, C. Moutinho, F.M. Nagengast, R. Seruca, H. van Krieken, F. Carneiro, Intragenic deletion of CDH1 as the inactivating mechanism of the wild-type allele in an HDGC tumour, Oncogene 23 (2004) 2236–2240.
[120] M. Barber, A. Murrell, Y. Ito, A.T. Maia, S. Hyland, C. Oliveira, V. Save, F. Carneiro, A.L. Paterson, N. Grehan, S. Dwerryhouse, P. Lao-Sirieix, C. Caldas, R.C. Fitzgerald, Mechanisms and sequelae of E-cadherin silencing in hereditary diffuse gastric cancer, J. Pathol. 216 (2008) 295–306.
[121] C. Oliveira, S. Sousa, H. Pinheiro, R. Karam, R. Bordeira-Carrico, J. Senz, P. Kaurah, J. Carvalho, R. Pereira, L. Gusmao, X. Wen, M.A. Cipriano, J. Yokota, F. Carneiro, D. Huntsman, R. Seruca, Quantification of epigenetic and genetic 2nd hits in CDH1 during hereditary diffuse gastric cancer syndrome progression, Gastroenterology 136 (2009) 2137–2148.
[122] W.M. Grady, J. Willis, P.J. Guilford, A.K. Dunbier, T.T. Toro, H. Lynch, G. Wiesner, K. Ferguson, C. Eng, J.G. Park, S.J. Kim, S. Markowitz, Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer, Nat. Genet. 26 (2000) 16–17.
[123] H. Peinado, E. Ballestar, M. Esteller, A. Cano, Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex, Mol. Cell. Biol. 24 (2004) 306–319.
[124] S. Fujii, A. Ochiai, Enhancer of zeste homolog 2 downregulates E-cadherin by mediating histone H3 methylation in gastric cancer cells, Cancer Sci. 99 (2008) 738–746.
[125] Q. Cao, J. Yu, S.M. Dhanasekaran, J.H. Kim, R.S. Mani, S.A. Tomlins, R. Mehra, B. Laxman, X. Cao, C.G. Kleer, S. Varambally, A.M. Chinnaiyan, Repression of E-cadherin by the polycomb group protein EZH2 in cancer, Oncogene 27 (2008) 7274–7284.
[126] C. Pena, J.M. Garcia, J. Silva, V. Garcia, R. Rodriguez, I. Alonso, I. Millan, C. Salas, A.G. de Herreros, A. Munoz, F. Bonilla, E-cadherin and vitamin D receptor regulation by SNAIL and ZEB1 in colon cancer: clinicopathological correlations, Hum. Mol. Genet. 14 (2005) 3361–3370.
[127] Y. Liu, Y. Hong, Y. Zhao, T.M. Ismail, Y. Wong, K.W. Eu, Histone H3 (lys-9) deacetylation is associated with transcriptional silencing of E-cadherin in colorectal cancer cell lines, Cancer Invest. 26 (2008) 575–582.
[128] J. von Burstin, S. Eser, M.C. Paul, B. Seidler, M. Brandl, M. Messer, A. von Werder, A. Schmidt, J. Mages, P. Pagel, A. Schnieke, R.M. Schmid, G. Schneider, D. Saur, E-cadherin regulates metastasis of pancreatic cancer *in vivo* and is suppressed by a SNAIL/HDAC1/HDAC2 repressor complex, Gastroenterology 137 (2009) 361–371 (371 e361-365).
[129] N. Herranz, D. Pasini, V.M. Diaz, C. Franci, A. Gutierrez, N. Dave, M. Escriva, I. Hernandez-Munoz, L. Di Croce, K. Helin, A. Garcia de Herreros, S. Peiro, Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor, Mol. Cell. Biol. 28 (2008) 4772–4781.
[130] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[131] L. He, G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation, Nat. Rev. Genet. 5 (2004) 522–531.
[132] G.J. Hurteau, J.A. Carlson, S.D. Spivack, G.J. Brock, Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin, Cancer Res. 67 (2007) 7972–7976.
[133] M. Korpal, E.S. Lee, G. Hu, Y. Kang, The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2, J. Biol. Chem. 283 (2008) 14910–14914.
[134] P.A. Gregory, A.G. Bert, E.L. Paterson, S.C. Barry, A. Tsykin, G. Farshid, M.A. Vadas, Y. Khew-Goodall, G.J. Goodall, The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1, Nat. Cell Biol. 10 (2008) 593–601.
[135] S.M. Park, A.B. Gaur, E. Lengyel, M.E. Peter, The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2, Genes Dev. 22 (2008) 894–907.
[136] H.X. Tan, Q. Wang, L.Z. Chen, X.H. Huang, J.S. Chen, X.H. Fu, L.Q. Cao, X.L. Chen, W. Li, L.J. Zhang, MicroRNA-9 reduces cell invasion and E-cadherin secretion in SK-Hep-1 cell, Med. Oncol. 27 (2010) 654–660.
[137] L. Ma, J. Young, H. Prabhala, E. Pan, P. Mestdagh, D. Muth, J. Teruya-Feldstein, F. Reinhardt, T.T. Onder, S. Valastyan, F. Westermann, F. Speleman, J. Vandesompele, R.A. Weinberg, miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis, Nat. Cell Biol. 12 (2010) 247–256.
[138] Y. Oki, J.P. Issa, Epigenetic mechanisms in AML—a target for therapy, Cancer Treat. Res. 145 (2010) 19–40.
[139] J. Simoes-Correia, J. Figueiredo, C. Oliveira, J. van Hengel, R. Seruca, F. van Roy, G. Suriano, Endoplasmic reticulum quality control: a new mechanism of E-cadherin regulation and its implication in cancer, Hum. Mol. Genet. 17 (2008) 3566–3576.
[140] R. Sitia, I. Braakman, Quality control in the endoplasmic reticulum protein factory, Nature 426 (2003) 891–894.
[141] B. Meusser, C. Hirsch, E. Jarosch, T. Sommer, ERAD: the long road to destruction, Nat. Cell Biol. 7 (2005) 766–772.
[142] J. van Hengel, F. van Roy, Diverse functions of p120ctn in tumors, Biochim. Biophys. Acta 1773 (2007) 78–88.
[143] A.U. Jawhari, M. Noda, M. Pignatelli, M. Farthing, Up-regulated cytoplasmic expression, with reduced membranous distribution, of the src substrate p120(ctn) in gastric carcinoma, J. Pathol. 189 (1999) 180–185.
[144] A.J. Karayiannakis, K.N. Syrigos, D. Alexiou, N. Kalahanis, T. Rosenberg, E. Bastounis, M. Pignatelli, Expression patterns of the novel catenin p120cas in gastrointestinal cancers, Anticancer. Res. 19 (1999) 4401–4405.
[145] G. Karatzas, A.J. Karayiannakis, K.N. Syrigos, E. Chatzigianni, S. Papanikolaou, G. Simatos, D. Papanikolaou, S. Bogris, Expression patterns of the E-cadherin–catenin cell–cell adhesion complex in gastric cancer, Hepatogastroenterology 47 (2000) 1465–1469.
[146] R. Apweiler, H. Hermjakob, N. Sharon, On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database, Biochim. Biophys. Acta 1473 (1999) 4–8.
[147] D.H. Dube, C.R. Bertozzi, Glycans in cancer and inflammation—potential for therapeutics and diagnostics, Nat. Rev. Drug Discov. 4 (2005) 477–488.
[148] S.S. Pinho, H. Osorio, M. Nita-Lazar, J. Gomes, C. Lopes, F. Gartner, C.A. Reis, Role of E-cadherin N-glycosylation profile in a mammary tumor model, Biochem. Biophys. Res. Commun. 379 (2009) 1091–1096.
[149] J. Iijima, Y. Zhao, T. Isaji, A. Kameyama, S. Nakaya, X. Wang, H. Ihara, X. Cheng, T. Nakagawa, E. Miyoshi, A. Kondo, H. Narimatsu, N. Taniguchi, J. Gu, Cell–cell interaction-dependent regulation of N-acetylglucosaminyltransferase III and the bisected N-glycans in GE11 epithelial cells. Involvement of E-cadherin-mediated cell adhesion, J. Biol. Chem. 281 (2006) 13038–13046.
[150] M. Yoshimura, Y. Ihara, Y. Matsuzawa, N. Taniguchi, Aberrant glycosylation of E-cadherin enhances cell–cell binding to suppress metastasis, J. Biol. Chem. 271 (1996) 13811–13815.
[151] R.H. Giles, J.H. van Es, H. Clevers, Caught up in a Wnt storm: Wnt signaling in cancer, Biochim. Biophys. Acta 1653 (2003) 1–24.
[152] A.B. Reynolds, A. Roczniak-Ferguson, Emerging roles for p120-catenin in cell adhesion and cancer, Oncogene 23 (2004) 7947–7956.
[153] G. Christofori, Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression, EMBO J. 22 (2003) 2318–2323.
[154] C.J. Gottardi, E. Wong, B.M. Gumbiner, E-cadherin suppresses cellular transformation by inhibiting beta-catenin signaling in an adhesion-independent manner, J. Cell Biol. 153 (2001) 1049–1060.

[155] A. Stockinger, A. Eger, J. Wolf, H. Beug, R. Foisner, E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity, J. Cell Biol. 154 (2001) 1185–1196.
[156] G. Suriano, D. Mulholland, O. de Wever, P. Ferreira, A.R. Mateus, E. Bruyneel, C.C. Nelson, M.M. Mareel, J. Yokota, D. Huntsman, R. Seruca, The intracellular E-cadherin germline mutation V832 M lacks the ability to mediate cell–cell adhesion and to suppress invasion, Oncogene 22 (2003) 5716–5719.
[157] E. Sahai, C.J. Marshall, RHO-GTPases and cancer, Nat. Rev. Cancer 2 (2002) 133–142.
[158] J. Deplazes, M. Fuchs, S. Rauser, H. Genth, E. Lengyel, R. Busch, B. Luber, Rac1 and Rho contribute to the migratory and invasive phenotype associated with somatic E-cadherin mutation, Hum. Mol. Genet. 18 (2009) 3632–3644.
[159] A.R. Mateus, R. Seruca, J.C. Machado, G. Keller, M.J. Oliveira, G. Suriano, B. Luber, EGFR regulates RhoA-GTP dependent cell motility in E-cadherin mutant cells, Hum. Mol. Genet. 16 (2007) 1639–1647.
[160] A.R. Mateus, J. Simoes-Correia, J. Figueiredo, S. Heindl, C.C. Alves, G. Suriano, B. Luber, R. Seruca, E-cadherin mutations and cell motility: a genotype–phenotype correlation, Exp. Cell Res. 315 (2009) 1393–1402.
[161] T. Crepaldi, A.L. Pollack, M. Prat, A. Zborek, K. Mostov, P.M. Comoglio, Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells, J. Cell Biol. 125 (1994) 313–320.
[162] S. Pece, J.S. Gutkind, Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell–cell contact formation, J. Biol. Chem. 275 (2000) 41227–41233.
[163] J.P. Thiery, Epithelial–mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (2002) 442–454.
[164] X. Qian, T. Karpova, A.M. Sheppard, J. McNally, D.R. Lowy, E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases, EMBO J. 23 (2004) 1739–1748.
[165] K. Takahashi, K. Suzuki, Density-dependent inhibition of growth involves prevention of EGF receptor activation by E-cadherin-mediated cell–cell adhesion, Exp. Cell Res. 226 (1996) 214–222.
[166] O.R. Mook, W.M. Frederiks, C.J. Van Noorden, The role of gelatinases in colorectal cancer progression and metastasis, Biochim. Biophys. Acta 1705 (2004) 69–89.
[167] H.G. Munshi, S. Ghosh, S. Mukhopadhyay, Y.I. Wu, R. Sen, K.J. Green, M.S. Stack, Proteinase suppression by E-cadherin-mediated cell–cell attachment in premalignant oral keratinocytes, J. Biol. Chem. 277 (2002) 38159–38167.
[168] A. Lochter, S. Galosy, J. Muschler, N. Freedman, Z. Werb, M.J. Bissell, Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells, J. Cell Biol. 139 (1997) 1861–1872.
[169] G. Davies, W.G. Jiang, M.D. Mason, Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell–cell dissociation and *in vitro* invasion, Clin. Cancer Res. 7 (2001) 3289–3297.
[170] V. Noe, B. Fingleton, K. Jacobs, H.C. Crawford, S. Vermeulen, W. Steelant, E. Bruyneel, L.M. Matrisian, M. Mareel, Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1, J. Cell Sci. 114 (2001) 111–118.
[171] F. Ryniers, C. Stove, M. Goethals, L. Brackenier, V. Noe, M. Bracke, J. Vandekerckhove, M. Mareel, E. Bruyneel, Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion, Biol. Chem. 383 (2002) 159–165.
[172] P.M. Comoglio, C. Boccaccio, L. Trusolino, Interactions between growth factor receptors and adhesion molecules: breaking the rules, Curr. Opin. Cell Biol. 15 (2003) 565–571.
[173] P. Ferreira, M.J. Oliveira, E. Beraldi, A.R. Mateus, T. Nakajima, M. Gleave, J. Yokota, F. Carneiro, D. Huntsman, R. Seruca, G. Suriano, Loss of functional E-cadherin renders cells more resistant to the apoptotic agent taxol *in vitro*, Exp. Cell Res. 310 (2005) 99–104.
[174] A.C. Ferreira, G. Suriano, N. Mendes, B. Gomes, X. Wen, F. Carneiro, R. Seruca, J.C. Machado, E-cadherin impairment increases cell survival through Notch-dependent upregulation of Bcl-2, Hum. Mol. Genet. 21 (2012) 334–343.
[175] F.Y. Huang, A.O. Chan, A. Rashid, D.K. Wong, C.H. Cho, M.F. Yuen, Helicobacter pylori induces promoter methylation of E-cadherin via interleukin-1beta activation of nitric oxide production in gastric cancer cells, Cancer (2012), http://dx.doi.org/10.1002/cncr.27519.
[176] A.O. Chan, S.K. Lam, B.C. Wong, W.M. Wong, M.F. Yuen, Y.H. Yeung, W.M. Hui, A. Rashid, Y.L. Kwong, Promoter methylation of E-cadherin gene in gastric mucosa associated with *Helicobacter pylori* infection and in gastric cancer, Gut 52 (2003) 502–506.
[177] W.K. Leung, E.P. Man, J. Yu, M.Y. Go, K.F. To, Y. Yamaoka, V.Y. Cheng, E.K. Ng, J.J. Sung, Effects of *Helicobacter pylori* eradication on methylation status of E-cadherin gene in noncancerous stomach, Clin. Cancer Res. 12 (2006) 3216–3221.
[178] F. Perri, R. Cotugno, A. Piepoli, A. Merla, M. Quitadamo, A. Gentile, A. Pilotto, V. Annese, A. Andriulli, Aberrant DNA methylation in non-neoplastic gastric mucosa of *H. pylori* infected patients and effect of eradication, Am. J. Gastroenterol. 102 (2007) 1361–1371.
[179] A.O. Chan, J.Z. Peng, S.K. Lam, K.C. Lai, M.F. Yuen, H.K. Cheung, Y.L. Kwong, A. Rashid, C.K. Chan, B.C. Wong, Eradication of *Helicobacter pylori* infection reverses E-cadherin promoter hypermethylation, Gut 55 (2006) 463–468.
[180] W. Schirrmeister, T. Gnad, T. Wex, S. Higashiyama, C. Wolke, M. Naumann, U. Lendeckel, Ectodomain shedding of E-cadherin and c-Met is induced by *Helicobacter pylori* infection, Exp. Cell Res. 315 (2009) 3500–3508.
[181] B. Hoy, M. Lower, C. Weydig, G. Carra, N. Tegtmeyer, T. Geppert, P. Schroder, N. Sewald, S. Backert, G. Schneider, S. Wessler, Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion, EMBO Rep. 11 (2010) 798–804.
[182] P.M. O'Connor, T.K. Lapointe, S. Jackson, P.L. Beck, N.L. Jones, A.G. Buret, *Helicobacter pylori* activates calpain via toll-like receptor 2 to disrupt adherens junctions in human gastric epithelial cells, Infect. Immun. 79 (2011) 3887–3894.
[183] V.S. Conlin, S.B. Curtis, Y. Zhao, E.D. Moore, V.C. Smith, R.M. Meloche, B.B. Finlay, A.M. Buchan, *Helicobacter pylori* infection targets adherens junction regulatory proteins and results in increased rates of migration in human gastric epithelial cells, Infect. Immun. 72 (2004) 5181–5192.
[184] M. Suzuki, H. Mimuro, T. Suzuki, M. Park, T. Yamamoto, C. Sasakawa, Interaction of CagA with Crk plays an important role in *Helicobacter pylori*-induced loss of gastric epithelial cell adhesion, J. Exp. Med. 202 (2005) 1235–1247.
[185] A.T. Franco, D.A. Israel, M.K. Washington, U. Krishna, J.G. Fox, A.B. Rogers, A.S. Neish, L. Collier-Hyams, G.I. Perez-Perez, M. Hatakeyama, R. Whitehead, K. Gaus, D.P. O'Brien, J. Romero-Gallo, R.M. Peek Jr., Activation of beta-catenin by carcinogenic *Helicobacter pylori*, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 10646–10651.
[186] N. Murata-Kamiya, Y. Kurashima, Y. Teishikata, Y. Yamahashi, Y. Saito, H. Higashi, H. Aburatani, T. Akiyama, R.M. Peek Jr., T. Azuma, M. Hatakeyama, *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells, Oncogene 26 (2007) 4617–4626.
[187] O. Sokolova, P.M. Bozko, M. Naumann, *Helicobacter pylori* suppresses glycogen synthase kinase 3beta to promote beta-catenin activity, J. Biol. Chem. 283 (2008) 29367–29374.
[188] S.R. Ogden, L.E. Wroblewski, C. Weydig, J. Romero-Gallo, D.P. O'Brien, D.A. Israel, U.S. Krishna, B. Fingleton, A.B. Reynolds, S. Wessler, R.M. Peek Jr., p120 and Kaiso regulate *Helicobacter pylori*-induced expression of matrix metalloproteinase-7, Mol. Biol. Cell 19 (2008) 4110–4121.
[189] J.R. Bebb, L. Leach, A. Zaitoun, N. Hand, D.P. Letley, R. Thomas, J.C. Atherton, Effects of *Helicobacter pylori* on the cadherin–catenin complex, J. Clin. Pathol. 59 (2006) 1261–1266.
[190] M.J. Oliveira, A.M. Costa, A.C. Costa, R.M. Ferreira, P. Sampaio, J.C. Machado, R. Seruca, M. Mareel, C. Figueiredo, CagA associates with c-Met, E-cadherin, and p120-catenin in a multiproteic complex that suppresses *Helicobacter pylori*-induced cell-invasive phenotype, J. Infect. Dis. 200 (2009) 745–755.
[191] M.J. Oliveira, A.C. Costa, A.M. Costa, L. Henriques, G. Suriano, J.C. Atherton, J.C. Machado, F. Carneiro, R. Seruca, M. Mareel, A. Leroy, C. Figueiredo, *Helicobacter pylori* induces gastric epithelial cell invasion in a c-Met and type IV secretion system-dependent manner, J. Biol. Chem. 281 (2006) 34888–34896.
[192] A.C. Jang, M. Starz-Gaiano, D.J. Montell, Modeling migration and metastasis in *Drosophila*, J. Mammary Gland Biol. Neoplasia 12 (2007) 103–114.
[193] P.S. Pereira, A. Teixeira, S. Pinho, P. Ferreira, J. Fernandes, C. Oliveira, R. Seruca, G. Suriano, F. Casares, E-cadherin missense mutations, associated with hereditary diffuse gastric cancer (HDGC) syndrome, display distinct invasive behaviors and genetic interactions with the Wnt and Notch pathways in *Drosophila epithelia*, Hum. Mol. Genet. 15 (2006) 1704–1712.
[194] J. Caldeira, P.S. Pereira, G. Suriano, F. Casares, Using fruitflies to help understand the molecular mechanisms of human hereditary diffuse gastric cancer, Int. J. Dev. Biol. 53 (2009) 1557–1561.
[195] B. Humar, V. Blair, A. Charlton, H. More, I. Martin, P. Guilford, E-cadherin deficiency initiates gastric signet-ring cell carcinoma in mice and man, Cancer Res. 69 (2009) 2050–2056.
[196] G. Mbalaviele, C.R. Dunstan, A. Sasaki, P.J. Williams, G.R. Mundy, T. Yoneda, E-cadherin expression in human breast cancer cells suppresses the development of osteolytic bone metastases in an experimental metastasis model, Cancer Res. 56 (1996) 4063–4070.
[197] A.K. Perl, P. Wilgenbus, U. Dahl, H. Semb, G. Christofori, A causal role for E-cadherin in the transition from adenoma to carcinoma, Nature 392 (1998) 190–193.
[198] Y. Kuwabara, T. Yamada, K. Yamazaki, W.L. Du, K. Banno, D. Aoki, M. Sakamoto, Establishment of an ovarian metastasis model and possible involvement of E-cadherin down-regulation in the metastasis, Cancer Sci. 99 (2008) 1933–1939.
[199] Y. Shimoyama, S. Hirohashi, S. Hirano, M. Noguchi, Y. Shimosato, M. Takeichi, O. Abe, Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas, Cancer Res. 49 (1989) 2128–2133.
[200] D.S. Sanders, R. Bruton, S.J. Darnton, A.G. Casson, I. Hanson, H.K. Williams, J. Jankowski, Sequential changes in cadherin–catenin expression associated with the progression and heterogeneity of primary oesophageal squamous carcinoma, Int. J. Cancer 79 (1998) 573–579.
[201] A.S. Rocha, P. Soares, J.C. Machado, V. Maximo, E. Fonseca, K. Franssila, M. Sobrinho-Simoes, Mucoepidermoid carcinoma of the thyroid: a tumour histotype characterised by P-cadherin neoexpression and marked abnormalities of E-cadherin/catenins complex, Virchows Arch. 440 (2002) 498–504.
[202] M.A. Kim, E.J. Jung, H.S. Lee, H.E. Lee, H.K. Yang, D.Y. Oh, Y.J. Bang, W.H. Kim, P-cadherin expression in gastric carcinoma: its regulation mechanism and prognostic significance, Hum. Pathol. 41 (2010) 877–885.
[203] K. Imai, S. Hirata, A. Irie, S. Senju, Y. Ikuta, K. Yokomine, M. Harao, M. Inoue, T. Tsunoda, S. Nakatsuru, H. Nakagawa, Y. Nakamura, H. Baba, Y. Nishimura, Identification of a novel tumor-associated antigen, cadherin 3/P-cadherin, as a possible target for immunotherapy of pancreatic, gastric, and colorectal cancers, Clin. Cancer Res. 14 (2008) 6487–6495.
[204] L. Lo Muzio, G. Campisi, A. Farina, C. Rubini, G. Pannone, R. Serpico, G. Laino, A. De Lillo, F. Carinci, P-cadherin expression and survival rate in oral squamous cell carcinoma: an immunohistochemical study, BMC Cancer 5 (2005) 63.
[205] A.C. Han, M.I. Edelson, A. Peralta Soler, K.A. Knudsen, B. Lifschitz-Mercer, B. Czernobilsky, N.G. Rosenblum, H. Salazar, Cadherin expression in glandular tumors of the cervix, Cancer 89 (2000) 2053–2058.

[206] A. Longatto Filho, A. Albergaria, J. Paredes, M.A. Moreira, F. Milanezi, F.C. Schmitt, P-cadherin expression in glandular lesions of the uterine cervix detected by liquid-based cytology, Cytopathology 16 (2005) 88–93.
[207] I.M. Stefansson, H.B. Salvesen, L.A. Akslen, Prognostic impact of alterations in P-cadherin expression and related cell adhesion markers in endometrial cancer, J. Clin. Oncol. 22 (2004) 1242–1252.
[208] G. Moreno-Bueno, D. Hardisson, D. Sarrio, C. Sanchez, R. Cassia, J. Prat, J.G. Herman, M. Esteller, X. Matias-Guiu, J. Palacios, Abnormalities of E- and P-cadherin and catenin (beta-, gamma-catenin, and p120ctn) expression in endometrial cancer and endometrial atypical hyperplasia, J. Pathol. 199 (2003) 471–478.
[209] L. Quattrocchi, A.R. Green, S. Martin, L. Durrant, S. Deen, The cadherin switch in ovarian high-grade serous carcinoma is associated with disease progression, Virchows Arch. 459 (2011) 21–29.
[210] D. Sarrio, G. Moreno-Bueno, C. Sanchez-Estevez, I. Banon-Rodriguez, G. Hernandez-Cortes, D. Hardisson, J. Palacios, Expression of cadherins and catenins correlates with distinct histologic types of ovarian carcinomas, Hum. Pathol. 37 (2006) 1042–1049.
[211] A.P. Soler, G.D. Harner, K.A. Knudsen, F.X. McBrearty, E. Grujic, H. Salazar, A.C. Han, A.A. Keshgegian, Expression of P-cadherin identifies prostate-specific-antigen-negative cells in epithelial tissues of male sexual accessory organs and in prostatic carcinomas. Implications for prostate cancer biology, Am. J. Pathol. 151 (1997) 471–478.
[212] R.T. Bryan, P.A. Atherfold, Y. Yeo, L.J. Jones, R.F. Harrison, D.M. Wallace, J.A. Jankowski, Cadherin switching dictates the biology of transitional cell carcinoma of the bladder: ex vivo and *in vitro* studies, J. Pathol. 215 (2008) 184–194.
[213] I. Matos, R. Dufloth, M. Alvarenga, L.C. Zeferino, F. Schmitt, p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas, Virchows Arch. 447 (2005) 688–694.
[214] J.B. Arnes, J.S. Brunet, I. Stefansson, L.R. Begin, N. Wong, P.O. Chappuis, L.A. Akslen, W.D. Foulkes, Placental cadherin and the basal epithelial phenotype of BRCA1-related breast cancer, Clin. Cancer Res. 11 (2005) 4003–4011.
[215] J. Palacios, N. Benito, A. Pizarro, A. Suarez, J. Espada, A. Cano, C. Gamallo, Anomalous expression of P-cadherin in breast carcinoma. Correlation with E-cadherin expression and pathological features, Am. J. Pathol. 146 (1995) 605–612.
[216] A. Peralta Soler, K.A. Knudsen, H. Salazar, A.C. Han, A.A. Keshgegian, P-cadherin expression in breast carcinoma indicates poor survival, Cancer 86 (1999) 1263–1272.
[217] J. Paredes, F. Milanezi, J.S. Reis-Filho, D. Leitao, D. Athanazio, F. Schmitt, Aberrant P-cadherin expression: is it associated with estrogen-independent growth in breast cancer? Pathol. Res. Pract. 198 (2002) 795–801.
[218] A. Kovacs, J. Dhillon, R.A. Walker, Expression of P-cadherin, but not E-cadherin or N-cadherin, relates to pathological and functional differentiation of breast carcinomas, Mol. Pathol. 56 (2003) 318–322.
[219] C. Gamallo, G. Moreno-Bueno, D. Sarrio, F. Calero, D. Hardisson, J. Palacios, The prognostic significance of P-cadherin in infiltrating ductal breast carcinoma, Mod. Pathol. 14 (2001) 650–654.
[220] J. Paredes, A.L. Correia, A.S. Ribeiro, F. Milanezi, J. Cameselle-Teijeiro, F.C. Schmitt, Breast carcinomas that co-express E- and P-cadherin are associated with p120-catenin cytoplasmic localisation and poor patient survival, J. Clin. Pathol. 61 (2008) 856–862.
[221] W.D. Foulkes, I.M. Stefansson, P.O. Chappuis, L.R. Begin, J.R. Goffin, N. Wong, M. Trudel, L.A. Akslen, Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer, J. Natl. Cancer Inst. 95 (2003) 1482–1485.
[222] S.R. Lakhani, J.S. Reis-Filho, L. Fulford, F. Penault-Llorca, M. van der Vijver, S. Parry, T. Bishop, J. Benitez, C. Rivas, Y.J. Bignon, J. Chang-Claude, U. Hamann, C.J. Cornelisse, P. Devilee, M.W. Beckmann, C. Nestle-Kramling, P.A. Daly, N. Haites, J. Varley, F. Lalloo, G. Evans, C. Maugard, H. Meijers-Heijboer, J.G. Klijn, E. Olah, B.A. Gusterson, S. Pilotti, P. Radice, S. Scherneck, H. Sobol, J. Jacquemier, T. Wagner, J. Peto, M.R. Stratton, L. McGuffog, D.F. Easton, Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype, Clin. Cancer Res. 11 (2005) 5175–5180.
[223] J. Palacios, E. Honrado, A. Osorio, A. Cazorla, D. Sarrio, A. Barroso, S. Rodriguez, J.C. Cigudosa, O. Diez, C. Alonso, E. Lerma, L. Sanchez, C. Rivas, J. Benitez, Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to BRCA1 or BRCA2 mutations: differences from breast carcinomas arising in BRCA1 and BRCA2 mutation carriers, Clin. Cancer Res. 9 (2003) 3606–3614.
[224] J. Palacios, E. Honrado, A. Osorio, A. Cazorla, D. Sarrio, A. Barroso, S. Rodriguez, J.C. Cigudosa, O. Diez, C. Alonso, E. Lerma, J. Dopazo, C. Rivas, J. Benitez, Phenotypic characterization of BRCA1 and BRCA2 tumors based in a tissue microarray study with 37 immunohistochemical markers, Breast Cancer Res. Treat. 90 (2005) 5–14.
[225] J.J. Gorski, C.R. James, J.E. Quinn, G.E. Stewart, K.C. Staunton, N.E. Buckley, F.A. McDyer, R.D. Kennedy, R.H. Wilson, P.B. Mullan, D.P. Harkin, BRCA1 transcriptionally regulates genes associated with the basal-like phenotype in breast cancer, Breast Cancer Res. Treat. 122 (2010) 721–731.
[226] A. Albergaria, A.S. Ribeiro, S. Pinho, F. Milanezi, V. Carneiro, B. Sousa, S. Sousa, C. Oliveira, J.C. Machado, R. Seruca, J. Paredes, F. Schmitt, ICI 182,780 induces P-cadherin overexpression in breast cancer cells through chromatin remodelling at the promoter level: a role for C/EBPbeta in CDH3 gene activation, Hum. Mol. Genet. 19 (2010) 2554–2566.
[227] N. Sato, N. Fukushima, A. Maitra, H. Matsubayashi, C.J. Yeo, J.L. Cameron, R.H. Hruban, M. Goggins, Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays, Cancer Res. 63 (2003) 3735–3742.
[228] A. Milicic, L.A. Harrison, R.A. Goodlad, R.G. Hardy, A.M. Nicholson, M. Presz, O. Sieber, S. Santander, J.H. Pringle, N. Mandir, P. East, J. Obszynska, S. Sanders, E. Piazuelo, J. Shaw, R. Harrison, I.P. Tomlinson, S.A. McDonald, N.A. Wright, J.A. Jankowski, Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission *in vivo*, Cancer Res. 68 (2008) 7760–7768.
[229] V. Van Marck, C. Stove, K. Van Den Bossche, V. Stove, J. Paredes, Y. Vander Haeghen, M. Bracke, P-cadherin promotes cell–cell adhesion and counteracts invasion in human melanoma, Cancer Res. 65 (2005) 8774–8783.
[230] D. Sarrio, J. Palacios, M. Hergueta-Redondo, G. Gomez-Lopez, A. Cano, G. Moreno-Bueno, Functional characterization of E- and P-cadherin in invasive breast cancer cells, BMC Cancer 9 (2009) 74.
[231] K. Bauer, A. Dowejko, A.K. Bosserhoff, T.E. Reichert, R.J. Bauer, P-cadherin induces an epithelial-like phenotype in oral squamous cell carcinoma by GSK-3beta-mediated Snail phosphorylation, Carcinogenesis 30 (2009) 1781–1788.
[232] K. Bauer, A. Dowejko, A.K. Bosserhoff, T.E. Reichert, R. Bauer, Slit-2 facilitates interaction of P-cadherin with Robo-3 and inhibits cell migration in an oral squamous cell carcinoma cell line, Carcinogenesis 32 (2011) 935–943.
[233] J.A. Mandeville, B. Silva Neto, A.J. Vanni, G.L. Smith, K.M. Rieger-Christ, R. Zeheb, M. Loda, J.A. Libertino, I.C. Summerhayes, P-cadherin as a prognostic indicator and a modulator of migratory behaviour in bladder carcinoma cells, BJU Int. 102 (2008) 1707–1714.
[234] L.W. Cheung, P.C. Leung, A.S. Wong, Cadherin switching and activation of p120 catenin signaling are mediators of gonadotropin-releasing hormone to promote tumor cell migration and invasion in ovarian cancer, Oncogene 29 (2010) 2427–2440.
[235] A.B. Reynolds, J.M. Daniel, Y.Y. Mo, J. Wu, Z. Zhang, The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts, Exp. Cell Res. 225 (1996) 328–337.
[236] L. Baki, P. Marambaud, S. Efthimiopoulos, A. Georgakopoulos, P. Wen, W. Cui, J. Shioi, E. Koo, M. Ozawa, V.L. Friedrich Jr., N.K. Robakis, Presenilin-1 binds cytoplasmic epithelial cadherin, inhibits cadherin/p120 association, and regulates stability and function of the cadherin/catenin adhesion complex, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 2381–2386.
[237] K. Jacobs, M. Van Gele, R. Forsyth, L. Brochez, B. Vanhoecke, O. De Wever, M. Bracke, P-cadherin counteracts myosin II-B function: implications in melanoma progression, Mol. Cancer 9 (2010) 255.
[238] G.L. Radice, C.L. Sauer, I. Kostetskii, A. Peralta Soler, K.A. Knudsen, Inappropriate P-cadherin expression in the mouse mammary epithelium is compatible with normal mammary gland function, Differentiation 71 (2003) 361–373.
[239] D.J. Gregory, L. Mikhaylova, A.V. Fedulov, Selective DNA demethylation by fusion of TDG with a sequence-specific DNA-binding domain, Epigenetics 7 (2012).
[240] C.C. Zhang, Z. Yan, Q. Zhang, K. Kuszpit, K. Zasadny, M. Qiu, C.L. Painter, A. Wong, E. Kraynov, M.E. Arango, P.P. Mehta, I. Popoff, G.F. Casperson, G. Los, S. Bender, K. Anderes, J.G. Christensen, T. VanArsdale, PF-03732010: a fully human monoclonal antibody against P-cadherin with antitumor and antimetastatic activity, Clin. Cancer Res. 16 (2010) 5177–5188.